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TITLE: A Cohort Study of the Relationship Between c-erbB-2 and Cyclin D1 Overexpression, p53 Mutation and/or Protein Accumulation, and Risk of Progression from Benign Breast Disease to Breast Cancer; and Creation of a Bank of Benign Breast Tissue

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13. ABSTRACT (Maximum 200 Words) Previously, in a case-control study, nested within a cohort of 4,888 women with BBD, we demonstrated that p53 protein accumulation detected by immunohistochemistry was associated with a 2.5-fold increase in the risk of subsequent breast. The purpose of this project was threefold: 1) to collect paraffin-embedded benign breast tissue from the remaining cohort members who were not part of the original case-control study. 2) enlarge the completed case-control study of p53 with an additional cases and matched controls. The tissue biopsies from these subjects were examined for c-erbB-2 overexpression and p53 protein accumulation. For some subjects breast tissue is being examined for the presence of p53 mutations. 3) to examine whether cyclin D1 amplification and/or protein overexpression determined immunohistochemically is a molecular marker of risk of progression from BBD to breast cancer in the enlarged case-control study. The studies supported by this grant demonstrated that 1) cyclin D1 protein overexpression occurs in normal and benign breast tissue; 2) cyclin D1 gene amplification and protein overexpression as detected immunohistochemically were not associated with increased risk of developing breast cancer in the group of individuals studied to date; 3) p53 mutations can occur in benign breast tissue. Also a tissue bank of paraffin-embedded normal or benign breast tissue (total number of individuals:1900, total number of paraffin blocks: 2,954) was established.				
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INTRODUCTION

Previously we studied the associations between c-erbB-2 protein overexpression and p53 protein accumulation in benign breast tissue and the risk of subsequent breast cancer (1). The study was conducted as a case-control study nested within the cohort of 4888 women in the National Breast Screening Study of Canada (NBSS) who were diagnosed with benign breast disease and underwent active follow-up. Case subjects were the women who subsequently developed breast cancer (ductal carcinoma in situ (DCIS) or invasive carcinoma). Control subjects were matched to each case subject by NBSS study arm, screening center, year of birth, and age at diagnosis of benign breast disease. Accumulation of p53 protein was associated with an increased risk of progression to breast cancer (adjusted odds ratio (OR) = 2.55; 95% confidence interval (CI) = 1.01-6.40), whereas c-erbB-2 protein overexpression was not (adjusted OR = 0.65; 95% CI = 0.27-1.53). The findings for c-erbB-2 and p53 did not differ among strata defined by menopausal status, allocation within the NBSS, history of breast disease, and whether the benign breast disease was detected at a scheduled screen or between screens. The results were also similar after exclusion of case subjects whose diagnosis of breast cancer occurred within 1 year of their diagnosis of benign breast disease and after exclusion of subjects with DCIS. In summary, p53 protein accumulation, but not c-erbB-2 protein overexpression, appeared to be associated with an increased risk of progression to breast cancer in women with benign breast disease.

The purpose of this project was threefold. Specifically, we:

- (1) attempted to collect paraffin-embedded benign breast tissue from the remaining 4,336 cohort members who were not part of the case-control study. This was done to establish a bank of paraffin-embedded tissue for a cohort of women on whom there is extensive documentation of risk factor information.
- (2) enlarged the completed case-control study of p53. An additional 63 cases (and 5 matched controls per case) were identified as a result of a linkage of the NBSS database to the Canadian Cancer Database. The tissue biopsies from these subjects were examined for c-erbB-2 overexpression and p53 protein accumulation. Breast tissues from selected biopsies were examined for the presence of p53 mutations.
- (3) examined whether cyclin D1 amplification and/or protein overexpression determined immunohistochemically is a molecular marker of risk of progression from BBD to breast cancer in the enlarged case-control study.

BODY

STUDY DESIGN

This study used paraffin-embedded breast tissues, which have been obtained from the cohort of women enrolled within the National Breast Screening Study (NBSS) and who received a diagnosis of benign breast disease during the active follow-up phase of the study. In the ensuing paragraphs, we describe the NBSS first, and then present details of the collection of paraffin blocks.

(a) The National Breast Screening Study: The NBSS is a multi-center randomized controlled trial of screening for breast cancer in Canadian women aged 40 to 59 at recruitment (2,3). The study involves 89,835 women who were recruited at 15 screening centers across Canada. Recruitment commenced in 1980 and ended in 1985. Women were eligible to participate in the study if they had no history of breast cancer, were not currently pregnant, and had not had a mammogram in the preceding 12 months.

Women aged 40-49 years were randomized either to have annual mammography plus physical examination, or to have initial physical examination only, and women in both the intervention and the control group were taught breast self-examination. Randomization in the 50-59 year age group was either to annual mammography plus physical examination, or to annual physical examination alone (women in this arm of the 50-59 year age group were also taught breast self-examination).

(i) Diagnosis of BBD and breast cancer in the NBSS: At each visit, study participants had a physical examination. For those who were randomized to the intervention group, physical examination was followed by mammography, the films from which were read by a study radiologist who was unaware of the physical examination results. If the examiner or the radiologist reported an abnormality requiring further assessment, a referral was made to a review clinic where the participant was seen by a study surgeon. If, on review, a recommendation was made for biopsy, this recommendation was conveyed to the participant's family physician, and the participant was contacted and asked to visit her family physician for further management.

Women in both control groups were referred for mammography if either they or their primary care physician discovered an abnormality for which referral was warranted. Staff in each screening center identified the pathology laboratory in which biopsies were examined, and they obtained slides or blocks for review by a locally designated reference pathologist. Results of the histological review of the biopsies were forwarded to the coordinating center.

(ii) Follow-up in the NBSS: Follow-up continued until 1990. During this phase of the NBSS (when the study participants underwent the screening schedule corresponding to their allocation, as described in (a) above), there was in each study center a coordinator (usually a nurse) who had experience in clinic or study management. The coordinators were responsible for ascertaining whether the recommended diagnostic procedures had

been carried out and for collecting reports of the surgical and pathological procedures from the institutions where they had been performed. Procedures performed independently of the screening process were identified through annual questionnaires sent to study subjects, and reports of these procedures were then obtained from the relevant institutions. Following completion of their screening schedule, direct follow-up stopped for those with no diagnosis of breast cancer. However, until 1988-1990 (depending upon the province) information about new diagnoses of breast cancer was obtained by linkage with the provincial cancer registries (cancer is registered in each province in Canada, and, for Ontario at least, registration is essentially complete (4)). Subsequently, new diagnoses of cancer have been ascertained by linkage to the Canadian Cancer Database, which is operated by the Canadian Center for Health Information at Statistics Canada, and consists of registration data reported annually by the provincial registries. A linkage yielding incidence data to the end of 1993 and mid 2000 were done to yield additional years of follow-up data that **allowed** us to enlarge our case-control group.

(b) Description of the cohort: The immunohistochemical and molecular investigation is being undertaken within the cohort of 4,888 women within the NBSS who received a histopathologic diagnosis of BBD during the active follow-up phase of the NBSS. In order to reduce costs substantially while having relatively little impact on the precision of the estimates of association, the study is being conducted as a case-control study nested within this cohort. Cases are women who subsequently developed breast cancer, while controls are women who had not developed breast cancer by the date of diagnosis of the corresponding case. For the initial case-control study, five controls were selected for each case, and they were matched to the corresponding case on study arm within the NBSS, screening center, year of birth, and age at diagnosis of BBD.

Case definition: Cases are women with a history of BBD detected during the course of the NBSS who subsequently developed breast cancer.

Definition of controls: Controls are women who had not developed breast cancer by (but were alive at) the date of diagnosis of the corresponding case (they do, of course, have a diagnosis of BBD). Since there are no estimates of the likely magnitude of the effects of interest on risk of progression from BBD to breast cancer, we selected 5 controls for each case in order to maximize statistical power. Controls are matched as described above. These matching criteria are chosen either because the factors of interest are related to breast cancer risk (age, and possibly age at diagnosis of BBD) or because they are related to the risk of disease detection (allocation and screening centre). It is also conceivable that at least some of these factors are related to the exposures of interest. However, little is known about the "epidemiological" correlates of cyclin D1 and c-erbB-2 overexpression, and p53 protein accumulation. Additionally, the implicit matching on duration of follow-up (as well as age) means that the controls have had the same opportunity (at least, in terms of the elapse of time) to develop breast cancer as the cases.

Questionnaires: At the time of their enrolment in the NBSS, all participants completed a questionnaire which sought identifying information, as well as data on factors such as demographic characteristics, family history of breast cancer, menstrual and reproductive history, use of oral contraceptives and replacement estrogens, and cigarette smoking. Additionally, approximately two-thirds of the 89,835 women enrolled in the NBSS completed self-administered diet history questionnaires. The questionnaire also included questions on current and past height and weight, and on consumption of beer, wine, and spirits.

Technical Objective 1: Obtaining blocks from remaining cohort members

Methods:

(a) Coding, data entry, and processing: The lifestyle information was available on the computerized NBSS database. This was accessed and standard procedures for quality control were used for coding and data entry.

(b) Collection of paraffin-embedded breast material: A database consisting of identifying information, plus details of the location and accession number of the paraffin blocks to be collected was created. This information was used to generate lists for each hospital of the study participants for whom we wished to obtain paraffin blocks. We then wrote to the pathologist-in-chief at the hospital seeking the blocks.

Results and Conclusions:

Paraffin block acquisition: We contacted a total of 253 hospitals. The number has changed from our grant proposal because of the ongoing hospital mergers that are occurring in Canada. We obtained 2954 blocks from a total of 1900 subjects. Fifty-four hospitals (1153 blocks) have replied and informed us that the blocks requested have been discarded and 25 hospitals had sent the blocks to other locations and could not be retrieved.

Technical Objective 2: Assessing role of cyclin D1 in progression of BBD to breast cancer

Methods

Histopathological Review: Sections from blocks received for the expanded nested case-control study were reviewed and classified by Dr. Kandel and Dr. W. Hartwick, according to the criteria developed by Page (5), and as described in the consensus conference for DCIS (6). Briefly, in benign lesions, the presence or absence of epithelial proliferation was determined, and when epithelial proliferation was present, the lesions were classified further according to the presence or absence of cytological atypia. The cancers were classified by histological type.

Cell line controls: The human breast carcinoma derived cell lines ZR-75-1, which has two to five-fold amplification of cyclin D1 (7), MDA-MB-231, which shows no cyclin D1

gene amplification (8), and T47D, which shows cyclin D1 overexpression immunohistochemically (7), were obtained from the American Type Culture Collection. The cells were grown in culture, harvested using trypsin -EDTA, pelleted, and placed in 3% bacto-agar (Difco Laboratories, Detroit, MI), fixed in 10% buffered formalin and then embedded in paraffin. Five μ m thick sections were cut and used as controls for the polymerase chain reaction and/or immunostaining.

Cyclin D1 Immunostaining: Tissue sections which had been stored for up to three years underwent antigen retrieval and were incubated overnight at 4°C with antibody reactive with cyclin D1 protein (monoclonal, dilution 1:2000; Upstate Biotechnology, Lake Placid, NY). After washing, the sections were incubated with biotinylated antimouse IgG (dilution 1:200; Vector Laboratories) for 30 minutes at room temperature, followed by avidin-biotin peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories). Immunoreactivity was visualized with 3,3'-diaminobenzidine (Vector), and the sections were counterstained briefly with hematoxylin. T47D cells embedded in paraffin served as the positive control. The negative control consisted of replacing the primary antibody with Tris-buffered saline or nonimmune mouse serum (DAKO, Carpinteria, CA). Distinct nuclear staining in greater than 1% of epithelial cells indicated a positive reaction and cytoplasmic staining was considered nonspecific and interpreted as negative.

Cyclin D1 Gene Amplification:

Tissue microdissection: Five μ m sections were stained briefly with hematoxylin to visualize the epithelium. The sections were matched to the corresponding immunostained sections. The epithelium in the area of the tissue corresponding to that which had shown cyclin D1 immunoreactivity was microdissected out and placed in a microfuge tube. If the tissue had shown no immunostaining for cyclin D1 the corresponding section underwent random microdissection of epithelium. DNA was extracted by incubating the microdissected tissue in buffer (50 mM Tris-HCl, pH 8.5, 10 mM EDTA, 0.5% Tween 20) containing 0.5 mg/ml of proteinase K at 55°C for 48 hours. The proteinase K was then inactivated by heating at 95°C for 15 minutes.

Differential polymerase chain reaction: Semiquantitative differential polymerase chain reaction (PCR) was used to determine the presence of cyclin D1 gene amplification. Aliquots of the proteinase K digested tissue was examined for cyclin D1 amplification using PCR. Each run included DNA extracted from paraffin-embedded cell lines, MDA-MB-231 (negative control) and ZR-75-1 (positive control).

1 μ l of the digest was mixed with 14 μ l of PCR working solution containing 50 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 100 μ M of each dNTP, 1 U of AmpliTaq DNA polymerase and 0.4 μ M of each primer. The primers and PCR conditions are shown in the appended manuscript. The PCR products were separated on a 12% polyacrylamide gel at 200V for two hours and visualized following ethidium bromide staining. DNA from each sample was analyzed at least twice in separate polymerase chain reactions. Samples showing reproducible amplification of cyclin D1, then underwent a third PCR which included samples of DNA that had been extracted from the subject's breast stromal tissue. As cyclin D1 is not amplified in the stromal tissue, it served as an internal control for the presence of cyclin D1 amplification in the

breast epithelium. Direct sequencing of selected PCR products using the sense primer and the ThermoSequenase radiolabelled terminator cycle sequencing kit confirmed that the product was cyclin D1.

Quantification of cyclin D1 amplification: The ratio of the cyclin D1 PCR product to the DR PCR product was derived from photographic negatives of ethidium bromide stained gels. The bands were quantified by laser densitometry. There were at least two gels per PCR product and each gel was scanned two times. A mean ratio of cyclin D1 to DR of greater than 0.88 was considered indicative of gene amplification. This value was determined by identifying the point two standard deviations above the average of the ratios ($n=93$) obtained from the control cell line, MDA-MB-231, that had no gene amplification.

Results: Twelve cases and 29 controls showed cyclin D1 amplification. Gene amplification values ranged from 0.89 to 1.27. Previous studies have shown that the breast cell line ZR-75-1, which was used as positive control, had approximately a three-fold amplification and this cell line using our methodology had on average an amplification ratio of 1.26 ± 0.96 . This suggests that when cyclin D1 in the breast tissues was amplified it had at most three-fold amplification. After adjustment for confounding there was a statistically non-significant 40% increase in risk of breast cancer in association with cyclin D1 amplification.

Cyclin D1 protein overexpression was seen in 75 samples of benign breast disease. Fifteen of the immunopositive tissues were cases and 60 were controls. When the analysis was completed the presence of cyclin D1 overexpression was not associated with increased risk of developing breast cancer (unadjusted odds ratio = 1.07, 95% confidence intervals = 0.56-2.03).

Conclusion: This objective has been accomplished. The results are published in European Journal of Cancer Prevention (see appendix).

Technical Objective 3: Cyclin D1, p53 and cerbB-2 immunostaining, cyclin D gene amplification and p53 molecular analysis of additional cases and controls identified in 1999 linkage

Methods

Sixty-three cases were identified by this linkage. The appropriate controls (5 women/case) were selected.

Immunohistochemical staining for p53: 5 μ m sections were cut from the paraffin blocks, mounted on aminopropyltriethoxysilane (2%, Sigma Chemical Co. MO, USA) coated slides and deparaffinized. The sections underwent antigen retrieval (microwaved in 10 mM citrate buffer, pH 6.0, for 15 minutes at a medium high setting). In all sections, the endogenous peroxidase was inactivated using 3% hydrogen peroxide, and the sections blocked with normal goat serum (20 μ l/ml, Vector Laboratories, Burlingame, CA) containing 5% crystallized bovine serum albumin (BDH Laboratory Supplies, Poole, England) in phosphate buffered saline (PBS). The sections were incubated with antibody reactive with p53 (DO-7, monoclonal, 1:40 dilution; Novocastra Laboratories, Newcastle Upon Tyne, England). After washing, the sections were incubated with

biotinylated goat anti-mouse IgG (dilution 1:200, Vector Laboratories) for 30 minutes at room temperature followed by avidin-biotin peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories). Immunoreactivity was visualized with 3', 3-diaminobenzidine tetrahydrochloride (Vector Laboratories) and the sections counterstained briefly with hematoxylin. For p53, the positive controls will be sections from a paraffin-embedded breast cancer, which is known to have a p53 mutation associated with p53 protein accumulation. For the negative controls, the primary antibody was replaced with mouse non-immune serum.

For p53, any nuclear staining seen at 100x magnification was considered a positive reaction whereas cytoplasmic staining was considered nonspecific and interpreted as negative. The percentage of immunopositive cells was estimated and categorized into one of two groups: less than 10 percent of all epithelial cells or 10 percent or greater. Staining was considered localized if one duct with its associated ductules/lobules showed immunopositivity; all other positive immunostaining was considered diffuse. The slides were reviewed without knowledge of the case-control status of the study subjects.

Immunostaining for c-erbB-2: 5 μ m sections were cut from the paraffin blocks, mounted on aminopropyltriethoxysilane (2%, Sigma Chemical Co. MO, USA) coated slides and deparaffinized. The sections underwent antigen retrieval. The endogenous peroxidase was inactivated using 3% hydrogen peroxide, and the sections blocked with normal goat serum containing 5% crystallized bovine serum albumin in phosphate buffered saline (PBS). The sections were incubated overnight at 4°C with antibody reactive with c-erbB-2 (NCL-CB11, dilution 1:160). After washing, the sections were incubated with biotinylated goat anti-mouse IgG (dilution 1:200) for 30 minutes at room temperature followed by avidin-biotin peroxidase complex. Immunoreactivity was visualized with 3', 3-diaminobenzidine tetrahydrochloride and the sections counterstained briefly with hematoxylin. The positive control was sections from a paraffin-embedded breast cancer, which was known to have cerbB-2 amplification. For the negative controls, the primary antibody was replaced with mouse non-immune serum.

Cyclin D1 Immunostaining: This was performed as described in technical objective 2.

p53 molecular analysis: Five μ m sections were cut from the paraffin blocks and stored for up to 3 years. Prior to microdissection the sections were dewaxed and stained briefly with hematoxylin. The epithelium in the region of the tissue that had shown p53 immunoreactivity was microdissected out and placed in a microfuge tube. The tissue sections which showed no p53 protein accumulation immunohistochemically underwent random microdissection of epithelium. The tissue was digested with proteinase K (0.5 mg/ml in 50 mM Tris HCl, pH 8.5, 10 mM EDTA, 0.5% Tween 20) for at least 48 hrs at 55°C. The proteinase K was inactivated by heating at 95°C for 15 min.

An aliquot of the digest was amplified using PCR, [α -³³P]-dATP and exon-specific primers. An aliquot of the reaction product was separated on an 8% non-denaturing polyacrylamide gel and the gel was processed for autoradiography. Potential mutations were detected by shifts in band mobility. If no band shifts were detected in these samples, the tissue was considered to have no mutation. For samples showing band

shifts, the PCR-SSCP analysis was repeated. If the two PCR-SSCP analyses generated similar band shifts, it was sequenced. Negative controls including cells which contained no mutation and a blank water control were included in each analysis. In addition positive controls for exons 5 to 9 (exon 5:SKBr 3; exon 6:T47D; exon 7:colo 320 DM; exon 8: MDAMB468; exon 9: SW480) were also included where appropriate. The cell lines had been embedded in agar, fixed in 10% formalin, and paraffin-embedded to simulate the processing conditions of the breast tissue.

The DNA in the band excised from the SSCP gels was eluted into water. The DNA was reamplified by PCR using the same primers and the product was run on a 2% agarose gel. The band was extracted using QIAquick gel extraction kit (Qiagen Inc, Mississauga, ON). The purified DNA was sequenced using ThermoSequenase radiolabelled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, OH) and the sense primer according to the manufacturer's directions, followed by gel electrophoresis and autoradiography. Cell lines with known mutations were also included where appropriate. Gene alterations were compared to those listed for breast cancer in a p53 database (<http://www.iarc.fr/p53>).

Results: The tasks in this objective were dependent the identification of cases by the linkage of the NBSS database to the Canadian Cancer Database and this was delayed at Statistics Canada. Once the linkage was done the breast cancer diagnoses had to be verified and this took more time than anticipated for technical reasons. However, all this was completed and an additional 63 cases were identified and the controls were selected.

We developed the methods to detect p53 mutations in DNA extracted from paraffin embedded tissue (task 7). We analyzed all the p53 immunopositive breast tissues and 15 subjects whose breast tissue did not show p53 protein accumulation. p53 sequence changes occurred overall in 59.2% (16/27) of p53 immunopositive tissues. p53 mutations occurred in 33% (9/27) of immunopositive cases. Four (26.7%) of the immunonegative tissues showed gene alterations of which one was a mutation.

Conclusions: The results of the p53 molecular analysis have been published in the International Journal of Cancer (please see appendix).

As a result of the delay in the linkage the work to accomplish tasks 6 to 9 were completed but the statistical analysis and determination of the association with breast cancer risk has not yet been completed. We anticipate these should be done within 3 months.

Technical Objective 4: Linkage to the National Cancer Incidence Reporting System in calendar year 2000

Methods and Results

This objective entailed preparing a file for a second linkage to obtain patient clinical follow-up to the year 1998-2000. The file was prepared and transferred to Statistics Canada. They did the linkage and have identified additional cases. We have just received the output from that linkage.

Conclusion: This objective has been accomplished.

KEY RESEARCH ACCOMPLISHMENTS:

- creation of a tissue bank of benign breast tissue
- demonstrated that cyclin D1 protein overexpression and gene amplification occurs in normal and benign breast tissue
- demonstrated that cyclin D1 protein overexpression is not associated with increased breast cancer risk
- demonstrated that cyclin D1 gene amplification is not associated with increased breast cancer risk
- demonstrated that p53 mutations and gene changes occur in normal and benign breast tissue
- expansion of cohort study which should refine the role of p53 protein accumulation as a marker of increased breast cancer risk

REPORTABLE OUTCOMES:

A) The following publications, manuscripts, and abstracts have resulted from the work supported by this grant.

1. Zhu XL, Rohan T, Hartwick W, Kandel R. Cyclin D1 gene amplification and protein expression in benign breast disease and breast carcinoma. *Mod Pathol* 11: 1082-1088, 1998.
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B) Additional grants received based on these studies.

- 1) US Army Medical Research and Materiel Command (Are p53 mutations associated with increased risk of developing breast cancer? A molecular epidemiological study. #BC980784) that will allow the continuation of this project. This grant focuses on determining whether p53 gene changes are associated with increased risk of developing breast cancer in the entire cohort.

2) p53 in BBD and Breast Cancer Risk: A Multicenter Cohort

NIH

Principal Investigator: T. Rohan Co-investigators: C. Duggan, A.G. Glass, E.L. Harris, J. G. Jones, R. A. Kandel, A. Negassa, N. Olson, U. Raju, M.J. Worsham

C) With the support of this grant we have been able to establish a tissue bank of paraffin-embedded normal or benign breast tissue.

CONCLUSIONS

As the analysis of the results of the expanded case-control study is ongoing, the conclusions that can be drawn in terms of breast cancer risk are limited. The previously published case-control study involving the use of benign breast tissue from individuals enrolled in the NBBS has been called a "paradigm for future studies of additional biomarkers that may identify women with high risk benign breast disease" in a recent editorial about our studies (9). This supports the approach that we are using to identify biomarkers of increased breast cancer risk. We have further expanded the study group by combining with other centers in London (England), Portland and Detroit that have similar populations in order to do these studies in a more timely manner with enhanced statistical power.

The studies supported by this grant demonstrated that 1) cyclin D1 protein overexpression occurs in normal and benign breast tissue; 2) cyclin D1 gene amplification and protein overexpression as detected immunohistochemically were not associated with increased risk of developing breast cancer in the group of individuals studied to date; 3) p53 mutations can occur in benign breast tissue.

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APPENDIX

Revised Statement of Work

It is envisaged that the study will take approximately four years to complete, and it is proposed to have the study commence in 1996 (the first year of the grant period).

Technical Objective 1: Obtaining blocks from remaining 4,888 cohort members

Task 1: Months 1-48: Update data base

Task 2: Months 9-48: Acquisition of paraffin blocks for years 1980-1988

- a. Write to Directors of Laboratories at hospitals where paraffin blocks are stored.
- b. If no response, follow-up with phone calls.
- c. When blocks received, log details into database.
- d. Cut histological sections and extract DNA.

Technical Objective 2: Assessing role of cyclin D1 in progression of BBD to breast cancer.

Task 3: Months 2-10: Immunostaining for cyclin D1

- a. Immunostain with antibody reactive with cyclin D1.
- b. Detect positive reactions by light microscopy.

Task 4: Months 24 to 34: Cyclin D1 gene amplification

- a. Extract DNA.
- b. PCR: cyclin D1, interferon (housekeeping type gene).
- c. Polyacrylamide gels.
- d. Photograph polyacrylamide gels for negative images.
- e. Densitometry to quantify gene amplification.

Task 5: Months 35 to 37: Analysis and report preparation (cyclin D1).

- a. Add results of experimental work to the study database.
- b. Analyze data.
- c. Write scientific report.

Technical Objective 3: Cyclin D1, p53 and c-erbB-2 immunostaining, cyclin D1 gene amplification and p53 molecular analysis of additional cases and controls identified in 1999 linkage.

Task 6: Months 35 to 37: Immunostaining for c-erbB-2, p53, cyclin D1

Task 7: Months 38 to 42: p53 molecular analysis

- a. Extract DNA.
- b. PCR.
- c. SSCP.
- d. Sequencing of DNA if SSCP positive.

Task 8: Months 43 to 47: Cyclin D1 gene amplification

- a. Extract DNA.
- b. PCR: cyclin D1, interferon (housekeeping type gene).
- c. Polyacrylamide gels.
- d. Photograph polyacrylamide gels for negative images.
- e. Densitometry to quantify gene amplification.

Task 9: Month 48: Analysis and report preparation.

- a. Add results of experimental work to the study database.
- b. Analyze data.

Technical Objective 4: Linkage to the National Cancer Incidence Reporting System in calendar year 2000.

Task 10: Months 44-48: Prepare file for linkage and transfer file to Statistics Canada in Ottawa.

Cyclin D1 Gene Amplification and Protein Expression in Benign Breast Disease and Breast Carcinoma

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Cyclin D1 plays a critical role in regulating cell-cycle progression. Gene amplification and protein overexpression of cyclin D1 have been detected in breast cancer but little is known concerning whether these changes occur in normal breast tissue and in breast lesions associated with increased risk of development of invasive breast cancer. We looked for cyclin D1 gene amplification and protein overexpression in 30 cases of benign breast disease (16 epithelial hyperplasias without atypia and 14 atypical ductal hyperplasias) and 18 ductal carcinomas *in situ* by use of differential PCR and immunohistochemical staining. We compared the resulting frequencies to those in 15 cases of normal breast tissue and 17 invasive ductal carcinomas. We found *cyclin D1* gene amplification in 15% of those with normal breast tissue, 19% of those with epithelial hyperplasia without atypia, 27% of those with atypical ductal hyperplasia, 35% of those with ductal carcinoma *in situ*, and 25% of those with invasive ductal carcinoma; corresponding figures for protein overexpression were 13, 13, 57, 50, and 64%. These results suggest that *cyclin D1* amplification and protein overexpression can occur before histologic alterations are seen but that the frequencies of these changes are higher in histologic lesions with cellular atypia (atypical hyperplasia and ductal carcinoma *in situ*), reaching frequencies similar to those observed in invasive carcinoma.

KEY WORDS: Benign breast disease, *Cyclin D1*, Differential polymerase chain reaction, Immunohistochemistry.

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Cancer has been defined as a proliferative disorder characterized by unregulated cell growth (1). Under normal conditions, progression through the cell cycle is orderly and is regulated by cyclins and their associated cyclin-dependent kinases (cdk) (2, 3). Two major checkpoints exist, one at the G₁-S interface and a second at the G₂-M interface. The former prevents replication of damaged DNA, and the latter prevents segregation of structurally altered chromosomes (4). Disruption at either of these points might play a role in the pathogenesis of malignancy (1). Cyclin D is involved in regulating cell cycle progression from G₁ into the S phase (1). There are three types of cyclin D (D1, D2, and D3), each with its own pattern of tissue-specific expression. These cyclins can form complexes with cdk4 or cdk6, which then phosphorylate the retinoblastoma protein and allow the cells to pass into the S phase. Cells that overexpress cyclin D1 show reduced exit from G₁ to G₀ (quiescent phase), suggesting a role for cyclin D1 at this regulatory point (5). Several observations led to the suggestion that *cyclin D1* amplification and/or overexpression are tumorigenic. First, cell transformation results when *cyclin D1* is transfected with the adenovirus *E1A* oncogene into BRK cells (6). Second, rat fibroblasts transfected with *cyclin D1* have a shortened G₁ phase and form tumors when injected into nude mice (7). Third, mammary hyperplasia and breast cancer develop in transgenic mice that overexpress *cyclin D1* (8). Some researchers suggested that *cyclin D1* is not a dominant oncogene but one that requires the presence of other oncogenes to induce tumors (9), whereas other investigators suggested that *cyclin D1* overexpression enhances gene amplification and might contribute to genomic instability (10).

Cyclin D1 has been studied extensively in breast cancer. *Cyclin D1* amplification has been observed in as many as 33% of breast cancer cell lines and between 11 and 23% of human breast cancers (11-16). Cyclin D1 accumulation, detected immunohistochemically, occurs in as many as 81% of breast cancers, although the frequency seems to be de-

pendent on the antibody used (11, 12, 16). There are relatively few reports, however, of cyclin D1 in normal breast tissue and in breast lesions associated with increased risk of developing invasive breast cancer. Immunohistochemical studies showed that normal human breast epithelium and breast tissue adjacent to breast cancers demonstrate, at most, occasional cells that express cyclin D1 protein (14, 17, 18). An *in situ* hybridization study demonstrated that 18% of benign breast lesions showed cyclin D1 mRNA overexpression (19). *Cyclin D1* gene amplification and overexpression, as well as protein accumulation, also occur in ductal carcinoma *in situ* (DCIS) (20). The human and experimental data suggest that *cyclin D1* amplification and/or protein overexpression might have a role not only in breast cancer but also in the putative early stages of breast neoplasia, such as epithelial hyperplasia, a histopathologic change known to be associated with increased risk of progression to breast cancer (21–26). In this study, we explored the occurrence of cyclin D1 protein expression and gene amplification in a series of normal breast tissue samples, cases with benign breast disease, DCIS, and invasive ductal carcinoma.

MATERIALS AND METHODS

Tissues and Cell Lines

We searched the files of the Department of Pathology, Mount Sinai Hospital, Toronto, Ontario, Canada, for the period from 1990 to 1997 and identified 15 representative cases of normal breast tissue, 30 of benign breast disease (16 epithelial hyperplasias without atypia, 14 atypical ductal hyperplasias), 18 DCISs, and 17 breast carcinomas. The benign cases, as well as the DCISs, were from breast biopsy specimens that did not contain invasive carcinoma. The breast tissue had been fixed in 10% neutral buffered formalin and embedded in paraffin. The hematoxylin- and eosin-stained sections were reviewed by two of the authors (RK, WH) and classified according to the criteria described by Page *et al.* (27) and the 1997 consensus conference on the classification of DCIS (28). The following human breast carcinoma-derived cell lines:

- ZR-75-1 and MDA-MB-453, which have two-fold to fivefold amplification of *cyclin D1* (14);
- MCF-7, MDA-MB-468, and MDA-MB-231, which have no *cyclin D1* amplification (13, 14); and
- T47D, which shows cyclin D1 overexpression immunohistochemically (14)

were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in culture, harvested using trypsin-EDTA (Sigma, St.

Louis, MO), and centrifuged to form pellets. The cell pellets were placed in 3% bacto-agar (Difco, Detroit, MI), fixed in 10% buffered formalin, and then embedded in paraffin. Sections 5 μ m thick were cut and used as controls for polymerase chain reaction (PCR) and/or immunostaining.

Microdissection

Sections were cut from the paraffin blocks using standard precautions to avoid cross-contamination of tissue between cases. This included cutting one case at a time, changing microtome blades between cases, floating the section in its own water bath, and cleaning the work areas of the microtome with xylene between blocks. The sections were dried at 37° C overnight and then deparaffinized. The sections were stained with hematoxylin for 30 seconds, and the pathologic area was dissected with use of a dissecting light microscope (Laborlux 6000; Leica, Toronto, Canada). For the normal breast tissue sections, random ducts and/or lobules were microdissected. Mineral oil (4 μ L) (Sigma) was placed on the microdissected tissue, which was then transferred to a microfuge tube in a pipette tip.

DNA Extraction

Genomic DNA was extracted as described by Zhuang *et al.* (29), with some modifications. Briefly, the microdissected tissue was incubated in 50 μ L buffer (50 mM Tris-hydrochloric acid (HCl) (pH 8.5), 1 mM EDTA, 0.5% Tween 20) containing 0.5 mg/mL of proteinase K (Sigma) at 50° C for 48 hours. The proteinase K was then inactivated by boiling at 95° C for 15 minutes.

Cyclin D1 Amplification

Semiquantitative differential PCR was used to assess the presence of *cyclin D1* gene amplification and to estimate its extent. Fragmented genomic DNA (< 200 bp) can influence the results of differential PCR, so interferon- γ (IFN- γ) was analyzed in a multiplex PCR reaction to obtain an indirect assessment of DNA quality first (30, 31). Two sets of primers (Table 1), specific for different exons of the IFN- γ gene and which generate PCR products of 150 and 82 bp (IFN- γ 150, IFN- γ 82) were coamplified in the same reaction tube, as described previously (31). If the ratio of IFN- γ 82 to IFN- γ 150 in the PCR products was 3 or less, the tissue was considered suitable for additional analysis (31). For such cases, aliquots of the proteinase K-digested tissue were then examined by PCR for *cyclin D1* amplification. Both *asparagine synthetase* (*Asp*) and *cyclin D1* (Table 1) were coamplified in the same reaction tube. *Asp* is a housekeeping gene and served as an internal control. PCR was performed in

TABLE 1. Sequences of Polymerase Chain Reaction Primers

Gene	Sequences	Sequence region	Reference
IFN- γ 82	Sense, 5'-GCAGAGCCAAATGTCTCTCT-3'	(nt 2012-2031)	31
IFN- γ 82	Antisense, 5'-GGTCTCCACACTCTTTTGA-3'	(nt 2074-2093)	31
IFN- γ 150	Sense, 5'-TCTTTTCTTTCCCGATAGGT-3'	(nt 4582-4601)	31
IFN- γ 150	Antisense, 5'-CTGGGATGCTCTTCGACCTC-3'	(nt 4712-4731)	31
Cyclin D1	Sense, 5'-ATGTGAAGTTCATTTCCAAT-3'	(nt 722-741)	32
Cyclin D1	Antisense, 5'-TGGGTCACACTTGATCACTC-3'	(nt 851-870)	32
Asparagine synthetase	Sense, 5'-ACATTGAAGCACTCCGCGAC-3'	(nt 496-515)	44
Asparagine synthetase	Antisense, 5'-CCACATTGTCATAGAGGGCG-3'	(nt 639-658)	44

a total volume of 15 μ L. Briefly, 1 μ L of the digest was mixed with 14 μ L of PCR working solution containing 10 mM Tris-HCl (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.01% gelatin, 100 μ M of each dNTP, 1 U of AmpliTaq DNA polymerase (Roche Diagnostic Systems, Branchburg, NJ), and 1 μ M of each primer. The samples underwent 30 cycles of amplification in an automated thermocycler (DNA Thermal Cycler; Perkin Elmer, Branchburg, NJ). Each cycle consisted of 1.2 minutes of denaturation at 94° C (except for the first cycle, which was 10 minutes in length), 1 minute of annealing at 55° C, and 1 minute of elongation at 72° C. The PCR products were separated by electrophoresis on a 12% polyacrylamide gel at 200 V for 2 hours and visualized after ethidium bromide staining. Each tissue was analyzed at least twice in separate PCRs. Direct sequencing of PCR products of one control and case were performed with the initial sense primers and the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, OH), according to the manufacturer's directions.

Semiquantification of *Cyclin D1* Amplification

To assess whether *cyclin D1* gene amplification occurred, the ratio of the cyclin D1 PCR product to the *Asp* PCR product was derived from photographic negatives of ethidium bromide-stained gels that were quantified by laser densitometry (Computing Densitometer Model 300A; Molecular Dynamics, Sunnyvale, CA). There were at least two gels per sample, and each gel was scanned three times. A mean ratio of *cyclin D1* to *Asp* was calculated, and a ratio of greater than 1.5 was considered indicative of gene amplification (11, 32). The amount of *cyclin D1* gene amplification was categorized as + for a ratio of 1.5 through 2.5, ++ for a ratio between 2.5 and 3.5, and +++ for a ratio of more than 3.5.

Cyclin D1 Immunostaining

Tissue sections were placed on 2% aminopropyltriethoxysilane (Sigma)-coated slides and deparaffinized. The tissue underwent antigen retrieval (microwave pretreatment in 10 mM citrate buffer, pH 6.0, for 15 minutes at a medium-high setting). The

endogenous peroxidase was inactivated using 3% hydrogen peroxide, and the sections were blocked with normal horse serum (20 μ L/mL) Vector Laboratories, Burlingame, CA) containing 5% bovine serum albumin (BDH Laboratory, Poole, England) in Tris-buffered saline (5 mM Tris-HCl (pH 7.6) and 0.85% sodium chloride). The sections were incubated overnight at 4° C with antibody reactive with cyclin D1 protein (monoclonal, dilution 1:2000; Upstate Biotechnology, Lake Placid, NY). After washing, the sections were incubated with biotinylated antimouse immunoglobulin G (dilution 1:200; Vector) for 30 minutes at room temperature, followed by avidin-biotin peroxidase complex (Vectastain Elite ABC Kit; Vector). Immunoreactivity was visualized with 3,3'-diaminobenzidine (Vector), and the sections were counterstained briefly with hematoxylin. T47D cells embedded in paraffin served as the positive control (14). The negative control consisted of replacing the primary antibody with Tris-buffered saline or nonimmune mouse serum (DAKO, Carpinteria, CA). Distinct nuclear staining indicated a positive reaction and cytoplasmic staining was considered nonspecific and interpreted as negative. In normal tissue, the presence of staining in any of the epithelium was considered positive. In benign breast disease or cancer, only immunoreactivity in the pathologic area was interpreted as positive. The staining was assessed as + when only occasional cells were positive, ++ when clusters of cells were positive, and +++ when there was diffuse staining throughout the pathologic area.

RESULTS

Semiquantification of *Cyclin D1* Amplification

We used the cell lines, ZR-75-1, MDA-MB-453, MCF-7, MDA-MB-468, and MDA-MB-231, to assess the differential PCR assay for sensitivity and reproducibility. The *cyclin D1*-to-*Asp* ratio in the ZR-75-1 and MDA-MB453 cell lines, which according to Southern blot analysis have a twofold to fivefold amplification of *cyclin D1*, was always greater than 1.5. A ratio as high as 5.4 was obtained in some PCR runs. The other cell lines, which are not amplified for *cyclin D1*, had *cyclin D1*-to-*Asp* ratios of less than 1.5 in all runs. These results suggest that this

method is appropriate for determining whether the *cyclin D1* gene is amplified and sufficiently sensitive to detect twofold gene amplification. As shown in Figure 1, a 149-bp product consistent with cyclin D1 was detected in 13 normal samples, 16 hyperplasias, 11 atypical hyperplasias, 17 DCISs, and 16 carcinomas. PCR product sequencing was done for one control (ZR-75-1) and one case (Case C8), which confirmed that the product obtained was cyclin D1. Two normal samples (Cases N14, N15), three atypical hyperplasias (Cases A12, A13, A14), one DCIS (Case D18), and one invasive carcinoma (Case C17) exhibited poor DNA quality: no PCR products for either IFN- γ 82 or IFN- γ 150 were detected (results not shown). As detailed in Tables 2 and 3, amplification was detected in 2 (15%) of 13 cases of normal breast tissue, but, in both of these cases, the level of amplification was low. Three (19%) of 16 epithelial hyperplasias without atypia, 3 (27%) of 11 of atypical ductal hyperplasias, 6 (35%) of 17 DCISs, and 4 (25%) of 16 cancers showed gene amplification. The relative amount of amplification showed no correlation with the histologic changes, because only low levels of gene amplification were detected in breast cancer. Of the DCISs, one of four low-grade tumors, three of six intermediate-grade tumors, and two of seven high-grade tumors showed amplification.

Cyclin D1 Protein Overexpression

Protein overexpression was evaluated by immunohistochemical staining (Fig. 2). We saw cyclin D1 immunoreactivity in two cases (13%) of normal breast tissue (Tables 2 and 3) and two (13%) epi-

thelial hyperplasias without atypia. The proportion of cases showing protein accumulation was higher in the atypical hyperplasias (57%), in the DCISs (50%), and in the invasive cancers (64%) than in the cases of normal breast tissue and epithelial hyperplasia without atypia. In the normal breast tissue, only occasional cells were positive. The intensity and extent of immunostaining was more often greater in the cancers. Nonspecific cytoplasmic staining of epithelial cells, nerves, and/or blood vessels was seen in some sections, but this was easily distinguished from the nuclear staining indicative of protein overexpression.

Association of Gene Amplification and Protein Overexpression

Gene amplification occurred in the absence of protein overexpression and *vice versa* (Table 3). One of the 13 normal breast tissue samples had both gene amplification and protein overexpression, whereas 1 of the 16 epithelial hyperplasias without atypia, 2 of the 11 atypical ductal hyperplasias, 4 of the 17 DCISs, and 2 of the 16 cancers had both of these changes.

DISCUSSION

This study demonstrated that *cyclin D1* amplification and protein overexpression occur in normal tissue, breast tissue associated with increased breast cancer risk, and breast cancer. The frequencies of these changes were similar in normal tissue and epithelial hyperplasias without atypia but were higher in breast tissue showing atypical ductal hyperplasia and DCIS, reaching frequencies similar to those observed in invasive carcinoma. Experimental studies in transgenic mice showed that overexpression of cyclin D1 was associated with development of both hyperplasias and carcinomas (8). Our findings are in keeping with those results.

To date, three other studies examined cyclin D1 in benign breast disease in humans (19, 33, 34). Millikan *et al.* (33), whose study used differential PCR, did not demonstrate *cyclin D1* amplification in any of 60 subjects selected from a cohort of women with benign breast disease. There are two possible explanations for this discrepancy with our results. First, in their series, there were only 10 epithelial hyperplasias and 1 atypical ductal hyperplasia. Second, in our study, only tissue showing the specific pathologic change underwent molecular analysis, whereas in the study of Millikan *et al.* (33), localized molecular analysis was not performed. This could have decreased the sensitivity of their differential PCR, because the pathologic cells might have been diluted by noncontributory cells, such as stromal, endothelial, and inflammatory cells. In the second report, Weinstat-Saslow *et al.*

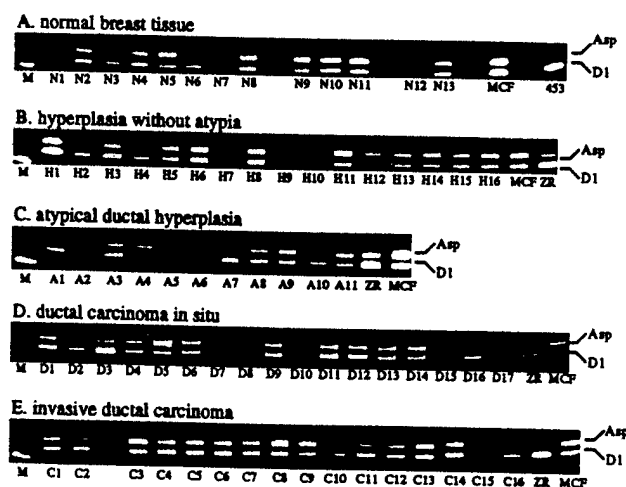


FIGURE 1. Ethidium bromide-stained gel showing PCR products from normal breast tissue (Cases N1-N13), epithelial hyperplasia without atypia (Cases H1-H16), atypical ductal hyperplasia (Cases A1-A11), DCIS (Cases D1-D17), invasive ductal carcinoma (Cases C1-C16), and controls. The PCR products for cyclin D1 (D1) and asparagine synthetase (Asp) are indicated. M, 123-bp DNA ladder. ZR, MCF, and 453 represent the human breast carcinoma-derived cell lines, ZR-75-1, MCF-7, and MDA-MB-453, which were the controls.

TABLE 2. Cyclin D1 Amplification and Protein Overexpression in Normal Tissue, Benign Breast Disease, and Breast Carcinoma

Normal breast tissue			Hyperplasia without atypia			Atypical ductal hyperplasia			Ductal carcinoma <i>in situ</i>			Invasive ductal carcinoma		
Case No.	AMP ^a	IHC ^b	Case No.	AMP ^a	IHC ^b	Case No.	AMP ^a	IHC ^b	Case No.	AMP ^a	IHC ^b	Case No.	AMP ^a	IHC ^b
N1	-	-	H1	-	-	A1	+++	++	D1	-	-	C1	-	+
N2	-	-	H2	++	-	A2	-	++	D2	+++	+	C2	+	+
N3	+	+	H3	-	-	A3	-	-	D3	++	++	C3	-	-
N4	-	-	H4	+	-	A4	-	-	D4	-	-	C4	-	-
N5	-	-	H5	-	-	A5	-	-	D5	-	-	C5	-	+
N6	+	-	H6	-	-	A6	-	+	D6	-	-	C6	-	+++
N7	-	-	H7	-	-	A7	+++	-	D7	-	+++	C7	-	+++
N8	-	-	H8	-	++	A8	-	++	D8	+++	-	C8	-	-
N9	-	-	H9	-	-	A9	-	-	D9	-	-	C9	-	+++
N10	-	-	H10	+++	++	A10	+	+	D10	++	+++	C10	+	-
N11	-	-	H11	-	-	A11	-	+	D11	-	-	C11	+	-
N12	-	-	H12	-	-	A12	NP ^c	++	D12	-	++	C12	-	++
N13	-	+	H13	-	-	A13	NP ^c	++	D13	-	++	C13	-	+++
N14	NP ^c	-	H14	-	-	A14	NP ^c	-	D14	-	+	C14	-	++
N15	NP ^c	-	H15	-	-				D15	+	-	C15	-	++
			H16	-	-				D16	+	+	C16	+	+++
									D17	-	-	C17	NP ^c	-
									D18	NP ^c	+			

^a Amplification of the *cyclin D1* gene as evaluated by different polymerase chain reaction scored as negative (-) or as +, ++, or +++, representing amplification between > 1.5 and ≤ 2.5, > 2.5 and ≤ 3.5, and > 3.5, respectively.

^b Immunohistochemical studies of cyclin D1, scored as negative (-) or as + for occasional positive cells, ++ for clusters of positive cells, or +++ for diffuse immunoreactivity.

^c No polymerase chain reaction product was detected.

TABLE 3. Association Between Cyclin D1 Gene Amplification and Protein Overexpression

Diagnosis	% Gene amplification ^a	% Protein overexpression ^b	% Amplified cases showing immunopositivity ^c	% Immunopositive cases showing amplification ^d
Normal breast	15 (2/13)	13 (2/15)	50 (1/2)	50 (1/2)
Hyperplasia without atypia	19 (3/16)	13 (2/16)	33 (1/3)	50 (1/2)
Atypical ductal hyperplasia	27 (3/11)	57 (8/14)	67 (2/3)	33 (2/6)
Ductal carcinoma <i>in situ</i>	35 (6/17)	50 (9/18)	67 (4/6)	50 (4/8)
Invasive ductal carcinoma	25 (4/16)	64 (11/17)	50 (2/4)	18 (2/11)

^a The numbers in parentheses indicate the number of amplified cases over the total number of cases that had a detectable polymerase chain reaction product.

^b The numbers in parentheses indicate the number of immunopositive cases over the total number of cases analyzed, including the cases which showed no polymerase chain reaction product.

^c The numbers in parentheses indicate the number of cases with both gene amplification and immunopositivity over the total number of cases with *cyclin D1* amplification.

^d The numbers in parentheses indicate the number of cases with both gene amplification and immunopositivity over the total number of immunopositive cases that had a detectable polymerase chain reaction product.

(19) examined cyclin D1 mRNA expression. It is not known whether cyclin D1 mRNA overexpression is the result of gene amplification, so their results are not directly comparable to those of this study. Those investigators, however, were able to show that cyclin D1 mRNA overexpression occurred in hyperplasias with or without atypia and that there was a higher frequency of overexpression in DCISs and invasive cancers than in the hyperplasias. The third report used only immunohistochemical methods to study cyclin D1 expression. In that study, Gillett *et al.* (34) demonstrated that eight of nine atypical duct hyperplasias showed immunostaining for cyclin D1. This is a higher frequency of positivity than we observed, but this discrepancy might have arisen because different antibodies were used in the two studies, an explanation supported by the fact that they also observed a higher percentage of DCISs with cyclin D1 immunopositivity than we did.

Cyclin D1 protein overexpression in breast cancer cells, as detected by immunostaining, was reported in 28 to 81% of cases (11, 12, 16, 35-38). Our findings are within this range: 64% of our cases showed protein overexpression. There were two previous studies examining cyclin D1 immunopositivity in DCIS. One report, in keeping with our study, showed that 50% of DCISs were immunopositive (20), whereas the other report (34) showed even a higher frequency. Also, in agreement with our findings, Bartkova *et al.* (17) showed cyclin D1 immunopositivity in occasional cells in normal breast epithelium. We observed protein overexpression in the absence of gene amplification, suggesting that other mechanisms, most likely post-transcriptional in nature, play a role in cyclin D1 protein overexpression, although it is possible detection of cyclin D1 immunohistochemically might not always be indicative of protein overexpression (34). Conversely, it is not evident why protein accumula-



FIGURE 2. Immunohistochemical detection of cyclin D1 protein in (A) florid epithelial hyperplasia without atypia and (B) invasive ductal carcinoma. Positive nuclear staining is present (3,3'-diaminobenzidine with hematoxylin counterstain; original magnification, 400 \times).

tion was not observed immunohistochemically in all of the cases with gene amplification. Other authors also observed similar discordances between gene amplification and protein overexpression (16, 20). Possible explanations include changes in protein and mRNA stability, increased transcriptional rate, and method and/or antibody insensitivity.

The fact that cyclin D1 amplification and protein overexpression were detected in normal breast tissue suggests that molecular and protein changes might occur before the development of histologic changes such as hyperplasia. It is possible that the presence of amplification was an artifact of the methodology or indicative of undetected aneuploidy. We consider these explanations unlikely, however, because several reports described molecular and protein changes in apparently normal breast tissue (39–41) and because the frequency of *cyclin D1* gene amplification in invasive carcinomas in this series was similar to that detected by others with use of Southern blot analysis, which is the standard methodology (11, 38).

Cyclin D1 changes were also detected in hyperplasias with and without atypia, histologic changes associated with increased risk of developing breast cancer. The frequency of these alterations was higher in the presence of cellular atypia. The difference between the two

groups was statistically significant for protein overexpression (Fisher's exact test, $P = .018$) but not for gene amplification (Fisher's exact test, $P = .662$). Our study was small, and we have no clinical follow-up, but our findings raise the question of whether positive staining for cyclin D1 will enhance our ability to predict breast cancer risk. This is of particular interest because the interobserver variability in the histologic diagnosis of atypical hyperplasia has led to questions concerning its usefulness as a marker of risk (42).

The role of cyclin D1 in the pathogenesis of breast cancer is not fully delineated despite intensive study. The low frequencies of gene amplification and protein overexpression in breast tissue showing no or minimal increased risk for breast cancer development, compared with those in breast tissue with a higher risk, suggest that changes in the *cyclin D1* gene and/or protein expression might play a role in malignant transformation. It is possible, however, that any such changes do not contribute directly to the malignant transformation of a cell but rather result in a phenotype that favors or allows the critical alterations to occur (43). For example, Zhou *et al.* (10) demonstrated that cyclin D1 overexpression in a transfected rat liver epithelial cell line resulted in increased number of cells with *CAD* gene amplification. A better assessment of the predictive significance of cyclin D1 changes in women with benign breast disease will come from prospective studies in which women with benign breast lesions are followed for the subsequent development of breast cancer. Studies of this type might also help to identify whether the presence or absence of gene amplification, the amount of gene amplification, and/or protein overexpression is the best predictor of risk of progression to breast cancer.

Acknowledgment: We thank Lori Cutler for her secretarial assistance.

Note added in proof: Since this manuscript was accepted for publication, there has been another report describing cyclin D1 protein overexpression in normal breast tissue and benign breast disease.

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Cyclin D1 protein overexpression and gene amplification in benign breast tissue and breast cancer risk

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Cyclin D1 amplification and/or protein overexpression have been observed not only in breast cancer but also in the putative early stages of breast neoplasia. In a case-control study nested within a cohort of 4888 women, we investigated whether the occurrence of cyclin D1 gene amplification and/or protein overexpression in benign breast tissue might identify women at increased risk of subsequent breast cancer development. Cases were 92 women with a histological diagnosis of benign breast disease who subsequently developed breast cancer. Five controls (women with benign breast disease who had not developed breast cancer by the date of diagnosis of the corresponding case) were selected randomly for each case from those non-cases available within strata defined by screening centre, National Breast Screening Study (NBSS) study arm, year of birth and age at diagnosis of benign breast disease. Paraffin blocks of benign tissue were suitable for immunostaining for 71 cases and 293 controls. Sufficient DNA for analysis was obtained from a total of 356 subjects (69 cases, 287 controls). The benign breast tissues and breast cancers were immunostained for cyclin D1 and also analysed for the presence of cyclin D1 gene amplification by differential polymerase chain reaction (PCR). Fifteen cases and 60 controls showed evidence of cyclin D1 immunostaining, and 12 cases and 29 controls showed cyclin D1 gene amplification. There was essentially no association between cyclin D1 protein overexpression in benign breast tissue and risk of subsequent breast cancer (adjusted odds ratio (OR) 1.06; 95% confidence interval (CI) 0.56–2.02). After adjustment for potential confounding, there was a statistically non-significant 40% increase in risk of breast cancer in association with cyclin D1 gene amplification (adjusted OR 1.41; 95% CI 0.62–3.22). As multiple genetic changes are required to develop breast cancer, it may not be until the cascade of molecular alterations leading to breast cancer development is understood that identification of biomarkers of breast cancer risk will be possible.

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Key words: Benign breast tissue, cyclin D1, gene amplification, protein overexpression.

Introduction

In non-tumorous cells, cyclin D1, together with the cyclins D2 and D3, is involved in regulating progression from G₁ to the S phase of the cell cycle (Barnes and Gillett, 1998; Steeg and Zhou, 1998). These cyclins form complexes with cdk4 or cdk6, which can then phosphorylate the retinoblastoma (Rb) protein, resulting in the release of E2F transcription factors

and allowing cells to progress into the S phase. Cyclin D1 has other regulatory effects. Specifically, it has been implicated in the replication and repair of DNA, as it can bind to PCNA (proliferating cell nuclear antigen) (Xiong *et al.*, 1992), a protein involved in DNA synthesis, and cells that overexpress cyclin D1 are unable to repair DNA damage induced by ultraviolet radiation (Pagano *et al.*, 1994). Experimentally, a transfected rat liver cell line that over-

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expresses cyclin D1 showed an increased number of cells with CAD amplification, suggesting that under specific conditions cyclin D1 may enhance gene amplification and contribute to genomic instability (Zhou *et al.*, 1996)

Given the multifarious effects of cyclin D1 on cell function and genomic integrity, it might be postulated that perturbations of normal cyclin D1 levels are related to altered cancer risk (Zhou *et al.*, 2000). Indeed, since cyclin D1 amplification and/or protein overexpression have been observed not only in breast cancer (Buckley *et al.*, 1993; Zhang *et al.*, 1994; Zukerberg *et al.*, 1995; Frierson *et al.*, 1996; Barbareschi *et al.*, 1997) but also in the putative early stages of breast neoplasia (Weinstat-Saslow *et al.*, 1995; Simpson *et al.*, 1997; Alle *et al.*, 1998; Gillett *et al.*, 1998; Zhu *et al.*, 1998), it might be postulated that such changes contribute to breast cancer development. Therefore, in the cohort study reported here, we investigated whether the occurrence of cyclin D1 gene amplification and/or protein overexpression in benign breast tissue might identify women at increased risk of subsequent breast cancer development.

Materials and methods

Subjects and methods

The study methods have been described in detail elsewhere (Rohan *et al.*, 1998). In brief, the investigation was undertaken as a case-control study nested within the cohort of 4888 women in the National Breast Screening Study (NBSS) who received a histopathological diagnosis of benign breast disease during the active follow-up phase of the NBSS. The NBSS is a multicentre randomized controlled trial of screening for breast cancer in 89 835 Canadian women who were recruited between 1980 and 1985, and who were followed actively until 1988 and passively thereafter (Miller *et al.*, 1981, 1992). Women were eligible to participate if they were 40–59 years old and had no previous history of breast cancer (*in situ* or invasive).

Diagnosis of breast disease in the NBSS. In the NBSS, patients with clinical or radiological evidence of a lesion underwent either needle aspirates or biopsies. For those subjects who had biopsies, the histological sections were reviewed for study purposes by a reference pathologist. The study reported here was re-

stricted to subjects who had no evidence of breast cancer (*in situ* or invasive) in their initial surgical biopsy as determined on review by an NBSS reference pathologist. Women with a history of previous benign breast disease were not excluded from participation. The characteristics of the cohort have been described previously (Rohan *et al.*, 1998).

Ascertaining outcome. Incident cases of breast cancer were ascertained by record linkage with the provincial cancer registries, and a death clearance was performed by linkage to the Canadian National Mortality Database (Miller *et al.*, 1992). The dates of the linkages varied by province, ranging from the end of 1988 to early 1991.

Definition of cases. Cases were the 92 women with a histological diagnosis of benign breast disease made by a reference pathologist during the active follow-up phase of the NBSS and who subsequently developed breast cancer. In this study, cancer was defined as any form of breast carcinoma; there were 16 cases with ductal carcinoma *in situ* (DCIS) and 76 cases with invasive carcinoma.

Definition and selection of controls. Controls were women with benign breast disease who had not developed breast cancer by (but were alive at) the date of diagnosis of the corresponding case. Five controls were selected randomly for each case from those non-cases available within strata defined by screening centre: NBSS study arm, year of birth and age at diagnosis of benign breast disease.

Questionnaires. At the time of their enrolment in the NBSS, all participants completed a questionnaire that sought data on potential breast cancer risk factors, including demographic characteristics, family history of breast cancer, and menstrual and reproductive history.

Acquisition of paraffin-embedded blocks of breast tissue. For the present study, hospitals and clinics storing the paraffin-embedded blocks of benign and malignant tissue were asked to send one representative block per lesion and to indicate the fixative type and whether the tissue had been frozen prior to fixation. Blocks or sections of paraffin-embedded benign tissue were obtained for 74 (80.4%) of the 92 cases and for 349 (75.9%) of the 460 controls; blocks or sections of malignant tissue were obtained for 62 (83.8%) of the 74 cases (Rohan *et al.*, 1998).

Histopathology review. Sections from the blocks received were reviewed and classified according to the criteria developed by Page and Anderson (1987) and the recent consensus conference (Schwartz *et al.*, 1997), without knowledge of the case-control status of the study subjects.

Breast cancer cell lines

The human breast carcinoma-derived cell lines ZR-75-1, which has two- to fivefold amplification of cyclin D1 (Bartkova *et al.*, 1994), MDA-MB-231, which shows no cyclin D1 gene amplification (Frierson *et al.*, 1996), and T47D, which shows cyclin D1 overexpression immunohistochemically (Bartkova *et al.*, 1994), were obtained from the American Type Culture Collection (ATCC). The cells were grown in culture, harvested using trypsin-EDTA (Sigma Chemical Co, St Louis, MO, USA), and centrifuged to form pellets. The cell pellets were placed in 3% bacto-agar (Difco Laboratories, Detroit, MI, USA), fixed in 10% buffered formalin and then embedded in paraffin. Sections (5 μ m) were cut and used as controls for the polymerase chain reaction (PCR) and/or immunostaining.

Cyclin D1 immunostaining. Immunostaining was performed as described previously (Zhu *et al.*, 1998). Briefly, tissue sections that had been stored for up to 3 years underwent antigen retrieval (microwave pretreatment in 10 mmol/l citrate buffer, pH 6.0, for 15 minutes at a medium-high setting) and were incubated overnight at 4°C with antibody reactive with cyclin D1 protein (monoclonal, dilution 1:2000; Upstate Biotechnology, Lake Placid, NY, USA). After washing, the sections were incubated with biotinylated anti-mouse immunoglobulin G (dilution 1:200; Vector Laboratories, Burlingame, California, USA) for 30 minutes at room temperature, followed by avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories). Immunoreactivity was visualized with 3,3'-diaminobenzidine (Vector Laboratories), and the sections were counterstained briefly with haematoxylin. T47D cells embedded in paraffin served as the positive control. The negative control consisted of replacing the primary antibody with Tris-buffered saline or non-immune mouse serum (Dako, Carpinteria, CA, USA). Distinct nuclear staining in more than 1% of epithelial cells indicated a positive reaction and cytoplasmic staining was considered non-specific and interpreted as negative.

Determination of cyclin D1 gene amplification

Tissue microdissection. Sections (5 μ m) were stained briefly with haematoxylin to visualize the epithelium. The sections were matched to the corresponding immunostained sections. The epithelium in the area of the tissue corresponding to that which had shown cyclin D1 immunoreactivity was microdissected out and placed in a microfuge tube. If the tissue had shown no immunostaining for cyclin D1 the corresponding section underwent random microdissection of epithelium. DNA was extracted by incubating the microdissected tissue in buffer (50 mmol/l Tris-HCl, pH 8.5, 10 mmol/l EDTA, 0.5% Tween 20) containing 0.5 mg/ml of proteinase K (Sigma Chemical Co) at 55°C for 48 h. The proteinase K was then inactivated by heating at 95°C for 15 minutes.

Differential polymerase chain reaction. Semiquantitative differential PCR was used to determine the presence of cyclin D1 gene amplification (Zhu *et al.*, 1998). As fragmented genomic DNA (< 200 bp) may influence the results of differential PCR, interferon γ (IFN γ), which is a single copy gene, was analysed in a multiplex PCR reaction in order to indirectly assess DNA quality first, as described previously (Frye *et al.*, 1989; Neubauer *et al.*, 1992; Zhu *et al.*, 1998). If the IFN γ 82/IFN γ 150 ratio of the PCR products was 3 or less, the tissue was considered suitable for further analysis. For such cases, aliquots of the proteinase K-digested tissue were then examined for cyclin D1 amplification using PCR. If chromosomal aneuploidy is present within the tissue it might simulate amplification, resulting in a false positive result. To prevent this we selected the dopamine receptor (DR) for co-amplification, as it is present on the same chromosome as cyclin D1 (Grandy *et al.*, 1989; Gramlich *et al.*, 1994), yet of sufficient distance from cyclin D1 that it is unlikely to be part of an amplified amplicon. Each run included DNA extracted from paraffin-embedded cell lines, MDA-MB-231 (negative control) and ZR-75-1 (positive control).

Briefly, 1 μ l of the digest was mixed with 14 μ l of PCR working solution containing 50 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.01% gelatin, 100 μ mol/l of each dNTP, 1 U of AmpliTaq DNA polymerase (Roche Diagnostic Systems Inc., Branchburg, New Jersey, USA) and 0.4 μ mol/l of each primer. The primers and PCR conditions are shown in Table 1. The PCR products were separated on a 12% polyacrylamide gel at 200 V for 2 hours and visualized following ethidium

bromide staining. DNA from each sample was analysed at least twice in separate polymerase chain reactions. Samples showing reproducible amplification of cyclin D1 then underwent a third PCR that included samples of DNA that had been extracted from the patient's breast stromal tissue. As cyclin D1 is not amplified in this tissue, it served as an internal control for the presence of cyclin D1 amplification in the breast epithelium. Direct sequencing of selected PCR products using the sense primer and the Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, Ohio, USA) confirmed that the product was cyclin D1 as described previously (Zhu *et al.*, 1998).

Quantification of cyclin D1 amplification. To determine whether there was cyclin D1 gene amplification, the ratio of the cyclin D1 PCR product to the DR PCR product was derived from photographic negatives of ethidium bromide-stained gels. The bands were quantified by laser densitometry (Computing Densitometer Model 300A, Molecular Dynamics, Sunnyvale, CA, USA). There were at least two gels per PCR product and each gel was scanned two times. A mean ratio of cyclin D1 to DR of greater than 0.88 was considered indicative of gene amplification. This value was determined by identifying the point two standard deviations above the average of the ratios ($n = 93$) obtained from the control cell line, MDA-MB-231, that had no gene amplification.

c-erbB-2 protein overexpression and p53 protein accumulation. Immunostaining for c-erbB-2 and p53 was performed as described previously (Rohan *et al.*, 1998).

Statistical analysis

Odds ratios (OR) and 95% confidence intervals (CI) for the associations between cyclin D1 protein overexpression and gene amplification and risk of breast cancer were obtained from conditional logistic regression models (Breslow and Day, 1980). Adjusted odds ratio estimates were obtained by including terms representing the following potential confounders in the regression models: history of breast cancer in a first-degree relative, age at menarche, age at first live birth, menopausal status (pre-, peri- and post-menopausal), body mass index [weight (kg)/height (m)²], and hyperplasia (ductal or lobular, with or without atypia). For categorical variables, tests for trend (on one degree of freedom) in associations were performed by fitting the categorized variables as continuous variables in conditional logistic regression models. Further analyses included within individual comparisons of cyclin D1 in benign breast disease and breast cancer. All statistical tests were two-sided.

Results

As described previously (Rohan *et al.*, 1998), blocks of benign tissue were obtained for 74 (80.4%) of the 92 cases and for 349 (75.9%) of the 460 controls; however, blocks were stained for 309 of the controls only, since for 40 controls benign tissue was not obtained for the corresponding case. For three cases and 15 controls, the benign tissue was inadequate for immunohistochemical analyses, and therefore the statistical analyses were based on 71 cases and 293 controls. DNA was extracted from paraffin blocks

Table 1. Primer sequences and PCR conditions

Primers	Sequences	Product size (bp)	PCR conditions
Cyclin D1	5'-ACCAGCTCCTGTGCTGCGAA-3' 5'-CAGGACCTCCTTCTGCACAC-3'	152	30 cycles, 95°C, 1 min, 55°C, 1 min, 72°C, 1 min
Dopamine receptor	5'-CCACTGAATCTGTCCTGGTATG-3' 5'-GCGTGGCATAGTAGTTGTAGTGG-3'	113	30 cycles, 95°C, 1 min, 55°C, 1 min, 72°C, 1 min
γ-IFN	5'-TCTTTTCTTTCCGATAGGT-3' 5'-CTGGGATGCTCTTCGACCTC-3'	150	30 cycles, 95°C, 1 min, 50°C, 1 min, 72°C, 1 min
γ-IFN	5'-GCAGAGCCAAATTGTCTCCT-3' 5'-GGTCTCCACACTCTTTTGGGA-3'	82	30 cycles, 95°C, 1 min, 50°C, 1 min, 72°C, 1 min

and yielded sufficient DNA suitable for analysis in a total of 356 subjects (69 cases, 287 controls).

As shown previously (Rohan *et al.*, 1998) in this study population, risk of breast cancer was altered little in association with a family history of breast cancer, age at menarche, age at first live birth, menopausal status, Quetelet's index, and the presence of hyperplasia in benign tissue. However, the patterns of risk were mostly in accord with expectation. Also, there were few differences between those subjects for whom benign tissue was and was not obtained with respect to their distributions by breast cancer risk factors.

Of the subjects whose benign tissue was suitable for immunohistochemical analysis, 15 cases and 60 controls showed evidence of cyclin D1 immunostaining (Figure 1 and Table 2). There was essentially no association between cyclin D1 protein overexpression in benign breast tissue and risk of subsequent breast cancer, and there was little variation in risk by the percentage of cells showing immunostaining. Twelve cases and 29 controls showed cyclin D1 gene amplification (Figure 2 and Table 2). After adjustment for potential confounding, there was a statistically non-significant 40% increase in risk of breast cancer in association with cyclin D1 gene amplification. For those with hyperplasia and cyclin D1 immunostaining, the adjusted OR was 1.66 (95% CI 0.75–3.71); for those with hyperplasia and cyclin D1 gene amplification, the adjusted OR was 1.55 (95% CI 0.52–4.69). Compared to those with neither cyclin D1 immunostaining nor cyclin D1 gene amplification, the adjusted OR (95% CI) for those with either or both of these changes was 1.47 (0.71–3.03)

and 0.99 (0.34–2.90), respectively. As oestrogens regulate cyclin D1 expression (Prall *et al.*, 1998) cyclin D1 levels might vary during the menstrual cycle. However, additional adjustment for days since last menstrual period had little effect on the odds ratio reported in Table 2 (data not shown).

After exclusion of the 19 cases (and their matched controls) whose diagnosis of breast cancer occurred within one year of their diagnosis of benign breast disease, the adjusted OR (95% CI) for the associations between cyclin D1 protein overexpression and gene amplification and risk of breast cancer were 1.15 (0.53–2.50) and 2.27 (0.90–5.71), respectively. When the analyses were restricted to the matched case-control sets containing cases with invasive breast cancer (that is, after exclusion of the 14 cases with DCIS and their matched controls), the adjusted OR for cyclin D1 immunopositivity was 1.29 (95% CI 0.58–2.87), while the adjusted OR for cyclin D1 gene amplification was 1.79 (95% CI 0.66–4.86). Also, when the 23 cases whose benign and malignant lesions occurred in opposite breasts were excluded, the adjusted ORs for cyclin D1 immunopositivity and gene amplification were 1.08 (95% CI 0.47–2.48) and 1.55 (95% CI 0.53–4.52), respectively. Also the results for cyclin D1 immunostaining and gene amplification did not differ between strata defined by age, menopausal status, NBSS study arm, history of previous breast disease, and whether the benign breast disease was screen-detected or interval-detected.

As described elsewhere (Rohan *et al.*, 1998), risk of breast cancer was increased in those with positive immunostaining for p53 in their benign breast tissue

Table 2. Association between cyclin D1 gene amplification or protein overexpression and risk of breast cancer

Aspect	Level	No. cases ^a	No. controls	Unadjusted OR (95% CI) ^b	Adjusted OR (95% CI) ^c
Immunostaining	Presence	Absent	56	1 ^d	1 ^d
		Present	15	1.07(0.56–2.03)	1.06 (0.56–2.02)
	% cells immunopositive	Absent	56	1 ^d	1 ^d
		< 10	11	1.14 (0.55–2.38)	1.08 (0.49–2.37)
		> 10	4	0.91 (0.29–2.89)	1.04 (0.31–3.48)
Gene amplification	Absent	57	258	1 ^d	1 ^d
	Present	12	29	1.67 (0.78–3.58)	1.41 (0.62–3.22)

^aUnmatched distributions (matched odds ratios cannot be calculated directly from these numbers).

^bAdjusted for matching factors only (using conditional logistic regression).

^cAdjusted for hyperplasia, age at menarche, age at first live birth, menopausal status, Quetelet's index and history of breast cancer in a first-degree relative.

^dReference category.

OR, odds ratio; CI, confidence interval.

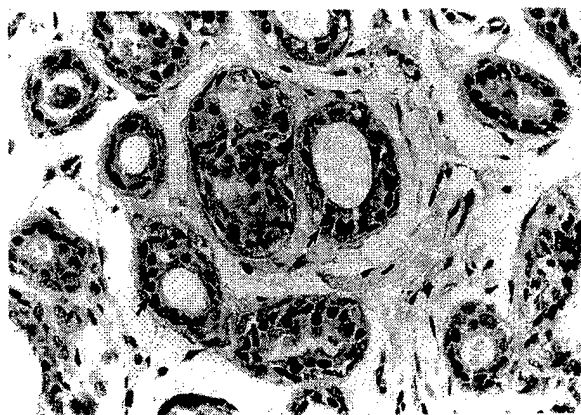


Figure 1. Nuclear immunostaining for cyclin D1 (↑) in scattered benign epithelial cells (immunoperoxidase with haematoxylin counterstain, magnification $\times 640$).

but not in those with immunostaining for c-erbB-2. When risk was examined according to the number of markers for which positive immunostaining was observed (relative to the risk in those with negative immunostaining for all three markers), the adjusted OR (95% CI) associated with positive immunostaining for one only and for two or more markers (only one case and one control had positive immunostaining for all three markers) were 1.19 (0.63–2.23) and 0.96 (0.33–2.81), respectively.

Table 3 shows the concordance between the immunohistochemical and gene amplification findings for the benign and malignant tissue for the cases. Of

the 39 subjects who were negative for cyclin D1 protein overexpression in their benign tissue, about 31% (12/39) showed evidence of overexpression in their malignant tissue; four (28.6%) of the 14 subjects with immunostaining in their benign tissue had cancers which did not show immunostaining. For gene amplification, the corresponding values were 21.2% (7/33) and 77.8% (7/9).

Discussion

Cyclin D1 gene amplification and/or protein expression has been detected in non-cancerous breast tissue. Breast epithelium that is either normal or has changes of benign breast disease, including breast papillomas, can show cyclin D1 protein overexpression immunohistochemically (Alle *et al.*, 1998; Gillett *et al.*, 1998; Zhu *et al.*, 1998; Saddik *et al.*, 1999). The frequency of cyclin D1 protein overexpression is greater in proliferative disease with atypia than in normal epithelium or in the presence of proliferative disease without atypia, as demonstrated in two studies (Alle *et al.*, 1998; Zhu *et al.*, 1998). In one of those studies (Zhu *et al.*, 1998), cyclin D1 gene amplification was also examined and was detected in normal and benign breast tissue. In an *in situ* hybridization study, 18% of benign breast lesions showed cyclin D1 mRNA overexpression (Weinstat-Saslow *et al.*, 1995). Cyclin D1 gene amplification and overexpression, as well as protein accumulation,



Figure 2. Ethidium bromide-stained gel of cyclin D1 (CD1) and dopamine receptor (DR) from 15 patients. For two patients, corresponding stromal tissue (S), which did not contain any breast epithelium, was also analysed as a control. ZR, ZR-75-1; MD, MDA-MB-231; 100bp = size ladder.

Table 3. Concordance between cyclin D1 immunostaining and gene amplification results for benign and malignant disease

Aspect	Percentage negative on benign and cancer (No.) ^{a,b}	Percentage negative on benign, positive on cancer (No.)	Percentage positive on benign, negative on cancer (No.)	Percentage positive on benign and cancer (No.)
Immunostaining	50.9 (27)	22.6 (12)	7.5 (4)	18.9 (10)
Gene amplification	61.9 (26)	16.7 (7)	16.7 (7)	4.8 (2)

^aPercentages are of all patients for whom both benign and malignant tissue was available.

^bOf the 62 cases for whom blocks of benign and malignant tissue were obtained, two blocks of benign tissue and seven of blocks of malignant tissue were unsuitable for analysis. For the analysis of gene amplification, the tissue was inadequate for analysis for seven cancers and no DNA was extracted from four cancers.

can also occur in ductal carcinoma *in situ* (DCIS) (Weinstat-Saslow *et al.*, 1995; Simpson *et al.*, 1997; Alle *et al.*, 1998; Gillett *et al.*, 1998; Zhu *et al.*, 1998) and breast cancer (Buckley *et al.*, 1993; Zhang *et al.*, 1994; Zukerberg *et al.*, 1995; Frierson *et al.*, 1996; Barbareschi *et al.*, 1997). In the latter, cyclin D1 accumulation, as detected immunohistochemically, has been observed in up to 81% of cases (Zukerberg *et al.*, 1995) and gene amplification has been observed in between 11 and 23% of cases (Lammie *et al.*, 1991; Zhang *et al.*, 1994; Courjal *et al.*, 1996; Frierson *et al.*, 1996; Worsley *et al.*, 1996).

In the present study cyclin D1 gene amplification and protein overexpression were detected in breast tissues at similar frequencies to those reported by others. However, we did not observe associations between the presence of either or both of these cyclin D1 alterations and breast cancer risk. Furthermore, the presence of either cyclin D1 amplification and/or protein overexpression in combination with epithelial hyperplasia was not associated with altered risk. There were too few cases of epithelial hyperplasia with atypia to permit a statistically meaningful evaluation of risk in association with this histological abnormality and cyclin D1 changes.

The benign breast tissue of 75 subjects showed positive immunostaining whereas the benign tissue of only 41 subjects showed gene amplification. The lack of correlation between cyclin D1 gene amplification and protein overexpression has been described previously (Frierson *et al.*, 1996; Pelosio *et al.*, 1996; Worsley *et al.*, 1996; Simpson *et al.*, 1997; Zhu *et al.*, 1998). It has been suggested that mechanisms other than gene amplification, such as post-transcriptional or post-translational mechanisms, could cause increased levels of cyclin D1 protein (Worsley *et al.*, 1996; Simpson *et al.*, 1997). For example, accumulation of cyclin D1 protein can occur because of transactivation of the cyclin D1 promoter by β -catenin (Lin *et al.*, 2000), or increased stability of the protein, as has been demonstrated in human uterine sarcomas (Welcker *et al.*, 1996), or decreased proteolysis, as has been shown to occur in the MCF-7 breast cancer cell line (Russell *et al.*, 1999). The mechanism(s) causing cyclin D1 protein overexpression in the absence of gene amplification in these breast tissues is unknown.

It is possible that the methodology used in this study influenced the results. The differential PCR assay used to determine whether gene amplification was present was assessed previously for sensitivity

and reproducibility (Zhu *et al.*, 1998). Although a different housekeeping gene was used in that study, the results suggested that differential PCR is appropriate for determining whether the cyclin D1 gene is amplified and that it is sufficiently sensitive to detect twofold gene amplification. In addition, other studies have utilized this approach for semi-quantitation of gene amplification (Nakagawa *et al.*, 1995; Schneeberger *et al.*, 1998; Suzuki *et al.*, 1998). In terms of the immunostaining, the methodology that was used may have resulted in an underestimation of the number of subjects overexpressing cyclin D1. It has been shown that the type and duration of tissue fixation, the sensitivity of the antibody, and the extent of tissue sampling may influence the sensitivity of immunohistochemistry (Elias, 1996; Rohan *et al.*, 1998). Also, given that the controls were women with biopsy-proven benign breast disease and that their risk for subsequent breast cancer is higher than that for women without benign breast disease (Page and Anderson, 1987) it is possible that we underestimated the magnitude of the association between cyclin D1 gene overexpression or protein accumulation and breast cancer risk.

Other than methodological limitations, there are several possible reasons why cyclin D1 changes were not associated with altered breast cancer risk. First, recent experimental data suggest that cyclin D1 is not a dominant oncogene but requires the presence of other oncogenes to form tumours (Barnes and Gillett, 1998). For example, transformation of BRK cells occurred when cyclin D1 was transfected together with the adenovirus E1A oncogene (Hinds *et al.*, 1994) and transformation of rat fibroblasts by cyclin D1 required the presence of Ha-ras (Lovec *et al.*, 1994). Also, transgenic mice containing cyclin D1 linked to an immunoglobulin enhancer rarely developed lymphoma until the mice were crossed with mice expressing the myc transgene (Bodrug *et al.*, 1994). Secondly, the quantification of cyclin D1 gene amplification suggested that the level of amplification in the benign tissue was in the range of that observed in the ZR-75-1 cell line, which shows two- to fivefold amplification. This low level of amplification may be insufficient to affect cell proliferation. Thirdly, the effect of cyclin D1 on the cell cycle is controversial, as stable transfection of cyclin D1 into HBL-100, a mammary epithelial cell line, resulted in longer doubling time, an increased percentage of cells in S phase, and decreased tumorigenesis (Han *et al.*, 1995). This is different from the effect observed for rat fibroblasts, suggesting that the effect of cyclin

D1 overexpression may be dependent on the cell type and which other genes are expressed (Jiang et al., 1993). Fourthly, the presence of increased cyclin D1 mRNA levels (Utsumi et al., 2000) or moderate to strong staining for cyclin D1 (Gillett et al., 1996) in breast cancer has been associated with a better prognosis, an observation that raises the possibility that cyclin D1 may not be involved in the pathogenesis of breast cancer.

In conclusion, in this study cyclin D1 amplification and/or protein overexpression in normal or benign breast tissue were not associated with increased risk of developing breast cancer. As experimental evidence suggests that cyclin D1 requires other oncogenes to induce tumorigenesis, assessment of cyclin D1 alterations alone may not be sufficient to identify women at increased risk of breast cancer. Instead, it may not be until the cascade of molecular alterations leading to breast cancer development (Beckmann et al., 1997; Ingvarsson, 1999) are known that the putative role of cyclin D1 in this process can be identified.

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p53 PROTEIN ACCUMULATION AND MUTATIONS IN NORMAL AND BENIGN BREAST TISSUE

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Mutations in the p53 gene are amongst the most common molecular changes detected in breast cancer, and there are several reports suggesting that changes in p53 may contribute to the pathogenesis of this disease. In a previous case-control study, we demonstrated that p53 protein accumulation detected by immunohistochemistry in normal or benign breast tissue was associated with a 2.5-fold increase in the risk of subsequent breast cancer. In this study, we investigated whether p53 gene mutations were present in the 29 p53 immunopositive normal or benign breast tissue samples and in 15 p53 immunonegative normal or benign breast tissue samples selected randomly from the original study. DNA was extracted from paraffin sections and underwent PCR-SSCP analysis for exons 4 to 10. PCR products that showed abnormal mobility were excised and sequenced. Sixteen (59.2%) of the 27 immunopositive breast tissue samples and 4 (26.7%) of the 15 immunonegative samples had p53 sequence changes. There was no obvious association between the occurrence of these alterations and any specific histopathologic features. Ten cases showed p53 mutations, and they were all missense base substitutions of the transition type. Thirteen other gene changes occurred in 11 breast tissue samples and consisted of 8 silent (no amino acid change), 4 intronic alterations, and 1 indeterminate alteration. One individual had both a mutation and a silent change. In summary, p53 gene alterations can occur in normal or benign breast tissue, but resolution of their role in the pathogenesis of breast cancer will require long-term follow-up studies involving comparisons of breast cancer occurrence in patients with and without p53 mutations as well as functional assays to determine their significance. *Int. J. Cancer* 87:73–78, 2000.

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Carcinogenesis is a complex multistep process that arises from the accumulation of critical genetic changes (Shackney and Shankey, 1997). The molecular changes leading to the development of breast cancer are not well characterized. However, mutations in the p53 gene are amongst the most common molecular changes detected in breast cancer (Phillips *et al.*, 1999) and several clinical and experimental studies have suggested that changes in p53 may contribute to the pathogenesis of this disease.

In experimental studies, p53 mutations occur in the preneoplastic stage of mouse mammary tumour development (Jerry *et al.*, 1993). It has been shown that transgenic mice expressing a mutant p53 172^{R-H} minigene that had been targeted to the mammary gland developed chemically induced breast cancer with shorter latency periods and greater tumour burden than did their nontransgenic littermates (Li *et al.*, 1998). Gao *et al.* (1996) have shown that ablation of p53 function by a dominant negative p53 mutant can result in immortalization of normal human mammary epithelial cells. However, not all dominant negative mutants induce immortalization (Gao *et al.*, 1997), suggesting that the contribution of mutant p53 to the development of cancer is complex.

In clinical studies, p53 mutations and/or p53 protein accumulation have been detected in intraductal carcinomas (Done *et al.*, 1998; Lisboa *et al.*, 1998; Phillips *et al.*, 1999). p53 protein accumulation has also been demonstrated immunohistochemically in the benign breast tissue of patients with the Li-Fraumeni syndrome (Thor *et al.*, 1992) and in benign tissue adjacent to breast cancer in women with a cancer syndrome distinct from Li-Frau-

meni syndrome (Barnes *et al.*, 1992). Several reports have also shown p53 mutations and/or positive immunostaining for p53 in sporadic forms of benign breast disease (Millikan *et al.*, 1995; Schmitt *et al.*, 1995; Younes *et al.*, 1995; Lisboa *et al.*, 1997; Rohan *et al.*, 1998). Collectively, these findings suggest that p53 changes can occur prior to the development of breast cancer. This is in keeping with observations by others that p53 alterations can occur in putative precursor lesions of other cancers and in normal tissues. For example, p53 mutations have been detected in Barrett's esophagus (Campomenosi *et al.*, 1996), and mutations in codons 247 and 248 have been detected in normal skin and have been shown to be associated with increased risk of developing basal cell carcinoma (Ouhthit *et al.*, 1998).

In a previous study (Rohan *et al.*, 1998) in which histological sections of normal or benign breast tissue were stained immunohistochemically for p53 (using the DO-7 antibody), we identified 29 subjects who showed p53 protein accumulation. One explanation for the p53 immunopositivity is that the tissue had an underlying p53 mutation. It is also possible that some of the 330 immunonegative subjects in that study had p53 mutations, since immunoreactivity can depend on the antibody used, on the type and duration of tissue fixation, or on the type of mutation, given that some mutations may not alter the protein in such a way that it can be detected immunohistochemically (Phillips *et al.*, 1999). In relation to the latter point, one study showed that approximately 33% of breast cancers with p53 gene mutations identified by complementary DNA sequencing did not show positive immunostaining in tissue sections using the C1 1801 antibody (Sjögren *et al.*, 1996). In this study, we investigated whether the 29 p53 immunopositive breast tissue samples and 15 randomly selected p53 immunonegative breast tissue samples had p53 gene mutations.

MATERIAL AND METHODS

Clinical history and histopathology review

Breast tissue specimens from 44 women whose biopsies showed either no histopathological change or benign breast disease were analyzed. The women selected for the study had their biopsies performed between 1980 and 1987. For each patient a representative paraffin block containing tissue from the breast biopsy was obtained. Five- μ m sections were cut, stained with hematoxylin and eosin, examined by light microscopy, and classified according to the criteria developed by Page and Anderson (1987).

p53 immunostaining

As described previously (Rohan *et al.*, 1998), 5- μ m sections were cut from the paraffin blocks, mounted on aminopropyltri-

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ethoxysilane (2%, Sigma, St. Louis, MO) coated slides and deparaffinized, and underwent antigen retrieval (microwaved in 10 mM citrate buffer, pH 6.0, for 15 min at a medium-high setting). Endogenous peroxidase was inactivated using 3% hydrogen peroxide, and the sections were blocked with goat serum (20 µl/ml, Vector, Burlingame, CA) containing 5% crystallized bovine serum albumin (BDH Laboratory Supplies, Poole, UK). The sections were incubated overnight at 4°C with antibody reactive with p53 (DO-7, dilution 1:40, Novocastra Laboratories, Newcastle Upon Tyne, UK). After washing, the sections were incubated with biotinylated goat anti-mouse IgG (dilution 1:200, Vector) for 30 min at room temperature, followed by avidin-biotin peroxidase complex (Vectastain Elite ABC Kit, Vector). Immunoreactivity was visualized with 3',3'-diaminobenzidine tetrahydrochloride (Vector) and the sections counterstained briefly with hematoxylin. The positive controls were sections from a paraffin-embedded breast cancer that was known to have a p53 mutation associated with p53 protein accumulation. The negative control consisted of replacing the primary antibody either with PBS or with mouse nonimmune serum. The presence of nuclear staining in any number of cells seen at 100× magnification was considered a positive reaction. Cytoplasmic staining was considered nonspecific and interpreted as negative.

p53 molecular analysis

Five-µm sections were cut from the paraffin blocks and stored for up to 3 years. Prior to microdissection, the sections were dewaxed and stained briefly with hematoxylin. The epithelium in the region of the tissue that had shown p53 immunoreactivity was microdissected out and placed in a microfuge tube. The tissue sections that showed no p53 protein accumulation immunohistochemically underwent random microdissection of epithelium. The tissue was digested with proteinase K (0.5 mg/ml in 50 mM Tris HCl, pH 8.5, 10 mM EDTA, 0.5% Tween 20) for at least 48 hr at 55°C. The proteinase K was inactivated by heating at 95°C for 15 min.

An aliquot of the digest was amplified using PCR, [α -³²P]-dATP and exon-specific primers (see Table I). An aliquot of the reaction product was separated on an 8% nondenaturing polyacrylamide gel, and the gel was processed for autoradiography. Potential mutations were detected by shifts in band mobility. If no band shifts were detected in these samples, the tissue was considered to have no mutation. For samples showing band shifts, the PCR-SSCP analysis was repeated. If the two PCR-SSCP analyses generated different band shifts, another section was cut, microdissected, and processed for PCR-SSCP analysis as described above. Negative controls including cells that contained no mutation and a blank water control were included in each analysis. In addition, positive controls for exons 5 to 9 (exon 5: SKBr 3; exon 6: T47D; exon 7: colo 320 DM; exon 8: MDAMB468; exon 9: SW480) were also included where appropriate. The cell lines used as positive

controls had been embedded in agar, fixed in 10% formalin, and were paraffin-embedded to simulate the processing conditions of the breast tissue.

The abnormally shifted band was excised from SSCP gels, and the DNA was eluted into water. The DNA was reamplified by PCR using the same primers, and the product was run on a 2% agarose gel. The band was extracted using QIAquick gel extraction kit (Qiagen, Mississauga, ON). The purified DNA was sequenced using ThermoSequenase radiolabelled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, OH) and the sense primer according to the manufacturer's directions, followed by gel electrophoresis and autoradiography. To confirm the mutation, the DNA product was resequenced using the antisense primer. Negative controls were included in each analysis. Cell lines with known mutations in exons 5 to 9 were also included where appropriate. Gene alterations were compared with those listed for breast cancer in a p53 database (<http://www.iarc.fr/p53>).

RESULTS

For two of the immunopositive cases, we were unable to extract DNA, and these cases were eliminated from the study. Of the 42 cases from which we could extract DNA, 22 showed fibrocystic change, 8 showed adenosis with or without fibrocystic change or fibrosis, 8 had hyperplasia (mild, moderate, or florid), 2 had fibroadenomas, and 2 showed no histopathological change.

Exons 4 to 10 were analyzed for mutations and a representative SSCP gel and its corresponding sequencing gel are shown in Figure 1. For all cases except one, the SSCP changes were reproducible. In the one case (case 24) where the SSCP change was not reproducible, the repeat analysis had been done on DNA extracted from a different section and only wild-type DNA sequences were seen on the second analysis. In all, 23 sequence alterations were detected in 20 individuals, and they were all base substitutions of the transition type (Tables II and III). Ten of these changes were missense mutations resulting in an amino acid change. Two of these mutations occurred at CpG dinucleotide sequences (cases 2 and 24), and 2 occurred at known hot spots on the p53 gene, one at codon 175 and the other at codon 245. The missense mutations were distributed amongst exons 4, 5, 7, and 9.

The other 13 gene changes (13/23) consisted of 8 silent (no amino acid change) base substitutions, 4 intronic base substitutions, and 1 uninterpretable change. The latter gene change occurred in case 5, for which an abnormal pattern for exon 9 was detected in the SSCP gel, while all the other exons showed wild-type patterns. Although the sequencing pattern could not be interpreted because of the presence of numerous extra bands, this sample was still considered to have a gene alteration. The 8 silent changes were detected in exons 4, 6, and 7, and the intronic changes were in introns 6, 7, and 9. Three individuals had 2 sequence changes each; in 2 of them both changes were silent and

TABLE I - P53 PRIMER SEQUENCES AND PCR CONDITIONS

Primers	Sequences	Product size (bp)	PCR conditions
Exon 4 ¹	5'-ATCTACAGTCCCCCTTGCCG-3' 5'-GCAACTGACCGTGCAAGTCA-3'	296 bp	95°C, 50 sec; 55°C, 50 sec; 72°C, 60 sec, 35 cycles
Exon 5 ²	5'-GCTGCCGTGTTCAGTTGCT-3' 5'-CCAGCCCTGTCGTCTCTCCA-3'	294 bp	95°C, 50 sec; 58°C, 50 sec; 72°C, 60 sec, 30 cycles
Exon 6 ²	5'-GGCCTCTGATTCCTCACTGA-3' 5'-GCCACTGACAACCACCCTTA-3'	199 bp	95°C, 50 sec; 55°C, 50 sec; 72°C, 60 sec, 30 cycles
Exon 7 ²	5'-TGCCACAGGTCTCCCCAAGG-3' 5'-AGTGTGCAGGGTGGCAAGTG-3'	196 bp	95°C, 50 sec; 56°C, 50 sec; 72°C, 60 sec, 30 cycles
Exon 8 ²	5'-CCTTACTGCCTCTTGCTTCT-3' 5'-ATAACTGCACCCTTGGTCTC-3'	225 bp	95°C, 50 sec; 55°C, 50 sec; 72°C, 60 sec, 30 cycles
Exon 9 ³	5'-GCCTCAGATTCACTTTATCACC-3' 5'-CTTCCACTTGATAAGAGGTCCCC-3'	152 bp	95°C, 50 sec; 56°C, 50 sec; 72°C, 60 sec, 30 cycles
Exon 10 ¹	5'-TTTGCTGCAGATCCGTGGG-3' 5'-GAGGTCACTCACCTGGAGTG-3'	130 bp	95°C, 50 sec; 55°C, 50 sec; 72°C, 60 sec, 33 cycles

¹Reference (Mashiyama *et al.*, 1991). ²Reference (Millikan *et al.*, 1995). ³Reference (Mazars *et al.*, 1992).

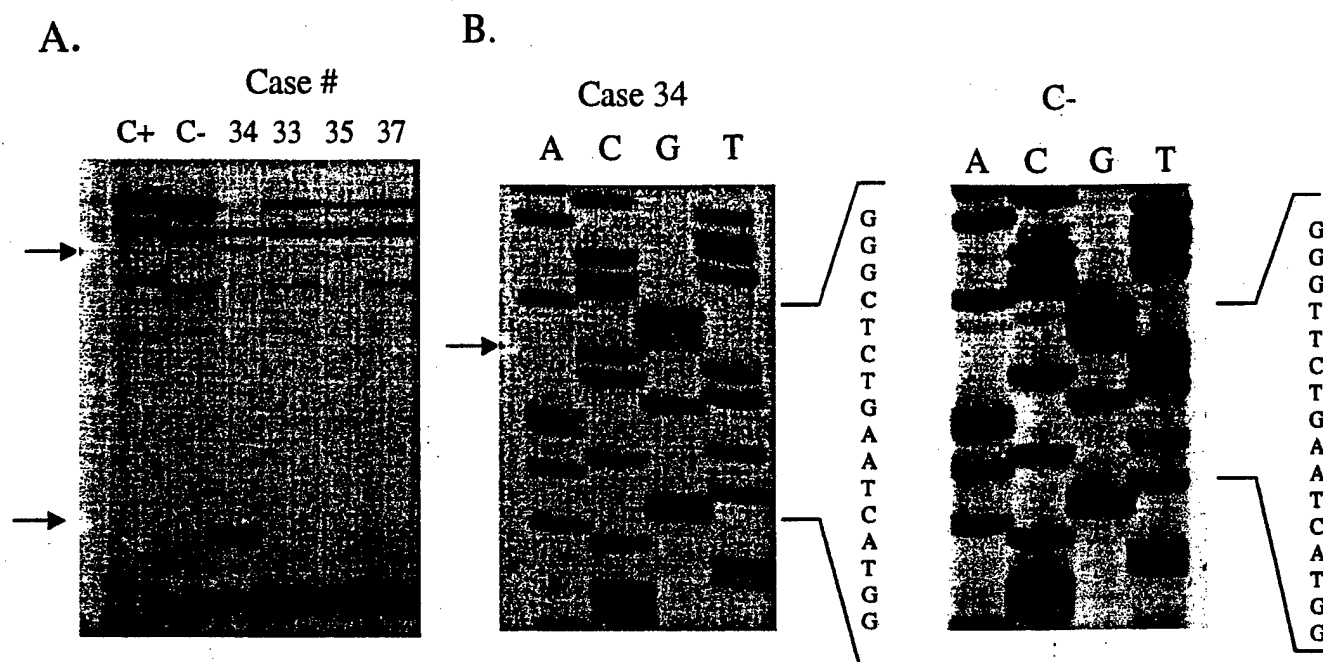


FIGURE 1 – (a) Representative SSCP gel of exon 9 PCR product from 4 cases. Case 34 shows a band shift as indicated by the arrow. The negative control that had wild-type p53 (C-) and positive (C+) control (cell line SW480) are included. (b) The corresponding sequencing gel of case 34 shows a base substitution (t→c) as indicated by the arrow. The sequencing pattern for the negative control (C-) in the same region is also shown.

TABLE II – P53 MUTATIONS IN BREAST TISSUE

Case number	p53 ICH ¹	Location	Codon	Sequence change	Amino acid
24	+	Exon 4	72	CGC → CGT	Arg → Arg
		Exon 4	110	CGT → TGT	Arg → Cys
46	+	Exon 4	76	GCA → ACA	Ala → Thr
2	+	Exon 5	175	CGC → CAC	Arg → His
4	+	Exon 5	135	TGC → TAC	Cys → Tyr
28	+	Exon 5	133	ATG → GTG	Met → Val
36	–	Exon 5	178	CAC → CGC	His → Arg
9	+	Exon 7	227	TCT → TTT	Ser → Phe
40	+	Exon 7	244	GGC → GAC	Gly → Asp
3	+	Exon 7	245	GGC → GAC	Gly → Asp
16	+	Exon 9	325	GGA → GAA	Gly → Glu

¹ICH, immunohistochemical staining; +, present, –, absent.

in the third, 1 of the 2 resulted in an amino acid change. Of the 4 intronic alterations, 2 were in the same location (nucleotide residue 14766) in intron 9 and showed the same change (t→c). The others were at nucleotides 13466 in intron 6 and 14114 in intron 7. None of the intronic mutations occurred at a splice site or created a new splice site. In addition to the results shown in Tables II and III, the known p53 polymorphism in codon 72 (CGC→CCC) was detected in 2 cases.

All 10 missense mutations occurred in codons identified in the p53 breast cancer database as having mutations. Seven of them showed the same base and amino acid change as has been identified in breast cancer. Of the 8 silent changes, 3 showed the same base change as has been identified in breast cancer and 5 showed a different alteration in the same codon. A similar comparison could not be done for the intronic mutations because the nucleotide residues of the intronic mutations are not provided in the database. Similar to those reported in the p53 database, most base substitutions in this study were G:A and C:T (IARC p53 mutations database <http://www.iarc.fr/p53>).

For all individuals with a p53 gene alteration, the adjacent stromal tissue underwent microdissection and extraction of the

DNA. The exon that had been identified as abnormal in the epithelial cells was analyzed by PCR-SSCP. In 18 of 20 samples/subjects, wild-type p53 banding patterns were observed (Fig. 2). In the other two (sample/subject 29 and 34), the same gene alteration was present in the stromal cells as in the epithelial cells.

Of the 27 breast tissue samples with p53 immunopositivity, 16 (59.2%) had p53 sequence changes. Nine of these 16 had mutations. One breast tissue with a p53 mutation was immunonegative (Table II). Seven of the 10 breast tissues with sequence alterations (silent or intronic) showed p53 immunoreactivity (Table III). Four of the 15 women (26.7%) whose biopsies were immunonegative showed sequence changes. One had a mutation (Table II) and 3 showed sequence alterations (Table III). There was no obvious association between the occurrence of gene alterations and any specific histopathologic features (Table IV). A representative photomicrograph of a section stained for p53 is shown in Figure 3.

DISCUSSION

p53 is involved in regulating cell proliferation and DNA repair, inducing apoptosis, and promoting chromosomal stability (Levine,

TABLE III - P53 SEQUENCE CHANGES THAT DO NOT CAUSE AMINO ACID CHANGES

Case number	p53 ICH ¹	Location	Site	Sequence	Amino acid
Silent change					
32	-	Exon 4	codon 74	GCC → GCT	Ala → Ala
		Exon 4	codon 111	CTG → CTA	Leu → Leu
44	+	Exon 4	codon 111	CTG → CTA	Leu → Leu
18	+	Exon 6	codon 217	GTG → GTA	Val → Val
27	+	Exon 7	codon 231	ACC → ACT	Thr → Thr
		Exon 7	codon 239	AAC → AAT	Asn → Asn
48	+	Exon 7	codon 226	GGC → GGT	Gly → Gly
Intronic change					
26	+	Intron 6	nr ² 13466	g → a	
17	+	Intron 7	nr 14114	g → a	
29	-	Intron 9	nr 14766	t → c	
34	-	Intron 9	nr 14766	t → c	
Noninterpretable change					
5	+	Exon 9	-	-	

¹IHC, immunohistochemical staining; +, present, -, absent. ²nr, nucleotide residue.

Case 44

Case 17

Case 16

Exon 4

Intron 7

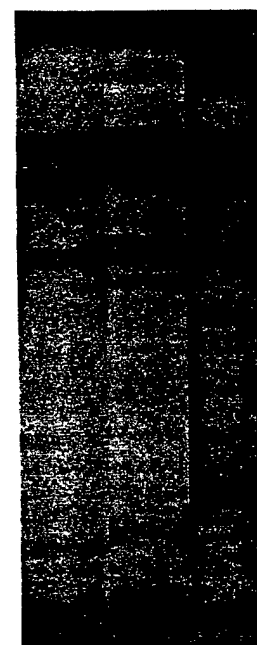
Exon 9



WT S E



WT S E



WT S E

FIGURE 2 - Representative SSCP gels of exon 4 (case 44), intron 7 (case 17), and exon 9 (case 16) showing DNA that had been extracted from epithelial cells (E), from corresponding stromal cells (S), and the appropriate negative control (WT). The DNA from the epithelial cells shows a different band pattern than the corresponding stromal and negative control DNA.

1997). Changes in p53 might contribute to carcinogenesis by conferring a proliferative advantage to cells with or without abnormal DNA and/or by facilitating the accumulation of additional genetic changes, for example by allowing aneuploidy and genetic instability to occur (Shackney and Shankey, 1997). To date, it is not known at which stage in the carcinogenic process p53 abnormalities develop (Phillips *et al.*, 1999).

Our results demonstrate that p53 gene alterations can be detected in breast tissue that is either normal or shows changes of

benign breast disease. p53 changes were found more commonly in tissue that showed p53 protein accumulation (positive immunostaining) than in tissue that did not. All of the changes detected were of the transition type. This is in keeping with experimental data showing that DNA proofreading corrects transversions more efficiently than transitions (Schaaper, 1993).

There have been 3 other reports of p53 gene analysis in normal or benign breast tissue. Millikan *et al.* (1995) detected p53 point mutations in 5 of 60 paraffin-embedded breast samples. Two of the

TABLE IV - SUMMARY OF ANALYSES OF p53 IMMUNOHISTOCHEMICALLY DETECTED PROTEIN ACCUMULATION AND GENE CHANGES ACCORDING TO HISTOLOGICAL FEATURES

Histology	Number of samples	Number of samples 1+ M+2	Number of samples 1+ M-	Number of samples 1- M+	Number of samples 1- M-
Normal	2	2	0	0	0
FCC ³	22	9	5	2	6
Adenosis ⁴	8	1	4	1	2
Hyperplasia ⁵	8	3	2	1	2
Fibroadenoma	2	1	0	0	1
Total	42	16	11	4	11

1+, immunopositive; 1-, immunonegative; 2M+, gene change present; M-, gene change absent; 3FCC, fibrocystic change; 4Adenosis, adenosis ± FCC ± fibrosis; 5Hyperplasia, either mild, moderate, or florid.



Figure 3 - Photomicrograph of normal breast ducts showing p53 immunopositivity (immunoperoxidase with hematoxylin counterstain, magnification 160x).

mutations occurred within the 14 cases that were immunopositive for p53 and the other 3 occurred in the 46 immunonegative cases. All of the mutations were transition types, and 3 resulted in an amino acid change. In contrast to our study, their analysis involved only exons 4 to 8, which may provide a partial explanation for the lower frequency of mutations in their study. Lisboa *et al.* (1997) detected a p53 mutation in one of 13 cases of normal or benign breast tissue examined. However, they examined only exons 5 to 8 inclusive and did not perform microdissection and so may have missed mutation(s) present in only a small number of cells. Done *et al.* (1998) identified 7 cases of breast cancer from which they were able to microdissect 41 foci of surrounding normal epithelium or epithelium showing changes of benign breast disease. The p53 gene analysis was performed on DNA extracted from paraffin-embedded tissue. They did not detect any mutations, but their study was based on a small number of cases and only exons 4 to 8 were studied.

In this study, sequence changes occurred overall in 59.2% (16/27) of p53 immunopositive samples. p53 mutations occurred in 33% (9/27) of immunopositive cases. Although this value may appear low, it is in keeping with the findings of several studies of breast cancer, which have examined the correlation between immunostaining and the presence of mutations detected by sequencing. In those studies, 20% (Dunn *et al.*, 1993) to 70% (Visscher *et al.*, 1996) of immunopositive breast cancers showed mutations. The relatively low value that we observed may in part reflect the fact that we considered the presence of any p53 immunopositivity to represent a positive case, whereas it has been suggested by others that only cases showing immunopositivity in greater than 5% of cells should be considered to have p53 protein accumulation (Clausen, 1998). It is also possible that more mutations may have been identified if the entire coding region (exons 2 to 11) and not

just exons 4 to 10 had been sequenced. Alternatively, the p53 protein accumulation may be due to mechanisms other than p53 mutation. Four (26.7%) of our p53 immunonegative cases showed gene alterations, one of which was a mutation. It is not surprising that p53 changes were detected in the absence of positive immunostaining as it is well accepted that not all p53 mutations will result in immunohistochemically detectable p53 protein (Sjögren *et al.*, 1996; Visscher *et al.*, 1996).

Several features of our study suggest that the mutations that were detected were real and were not artifacts of the methodology used to detect them. It has been shown that PCR-induced sequence changes can be minimized if the proteinase digestion time of the tissue is sufficiently prolonged (at least 48 hr), the products generated by PCR are relatively small (Shiao *et al.*, 1997), and enough DNA template is used (Krawczak *et al.*, 1989). In our study, the tissue was digested for at least 48 hr and the products were all less than 300 bp in size. Although we were unable to quantify the amount of DNA in each analysis, a fixed cycle number was used in the PCR for each exon of all samples and it was not increased if the product was undetectable. Secondly, repeat PCR-SSCP analysis showed that the band shifts were reproducible. In the one case where it was not reproducible, the analysis had been done on DNA extracted from a different section and it is likely that the area with the p53 change was no longer present. The DNA in the abnormal SSCP band was sequenced in both directions to ensure that the sequence change was not a PCR-induced artifact and in each case the same mutation(s) was detected. Thirdly, DNA from stromal tissue showed wild-type p53 sequences in 18 of 20 cases. The other 2 cases (29 and 34) had the same mutation in both epithelial and stromal DNA. For these samples, the changes might represent inadvertent microdissection of some epithelial cells with the stromal tissue or a true germline mutation or a polymorphism. We consider it more likely that the change detected in these two is a polymorphism because it has been detected in approximately 4% of tumours in a breast cancer tumour bank (data not shown). Fourthly, we were able to detect a known polymorphism in 2 other cases. Finally, other studies, such as that of Nadi *et al.* (1996), have shown that DNA extracted from paraffin-embedded tissue will show p53 gene changes identical to those detected in frozen tissue, suggesting that paraffin-embedding does not induce gene mutations and that tissue processed in this way is suitable for DNA analysis. Eight (34.8%) of the 23 gene changes detected were silent. Strauss (1997) predicted that approximately 25% of mutations in a dataset will be silent if mutagenesis is random and if the silent mutation does not provide a selective advantage. Although the significance of silent mutations is not known, it is possible that they could have effects on DNA.

In conclusion, the results of this study suggest that p53 mutations can be detected in normal epithelium and benign breast tissue. This observation is in keeping with the findings of other studies demonstrating genetic changes such as loss of heterozygosity (LOH) and microsatellite instability in normal and benign breast tissue (Deng *et al.*, 1996; Lakhani *et al.*, 1996; O'Connell *et al.*, 1998; Larson *et al.*, 1998). However, the significance of p53

mutations and other sequence changes in these tissue types is unknown. For example, a study showed that genetic changes such as LOH and microsatellite instability may not correlate with the development of breast cancer (Kasami *et al.*, 1997). For skin, however, it has been suggested that p53 mutations may provide information about subsequent risk of developing nonmelanoma skin cancer (Ouhitt *et al.*, 1998). Resolution of the role of p53 gene alterations in the pathogenesis of breast cancer may require long-

term follow-up studies involving comparisons of breast cancer occurrence in patients with and without p53 mutations and assessment of the functional significance of the mutations.

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Correlation of p53 Mutations in ThinPrep-Processed Fine Needle Breast Aspirates with Surgically Resected Breast Cancers

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Mutations of the p53 gene are one of the most common genetic changes found in cancer; their presence may be prognostic and even influence treatment for breast cancer. In this study, we investigated whether DNA could be extracted from the residual cells left in ThinPrep-processed breast fine-needle aspirates and whether p53 gene changes could be detected in the DNA. The results were then correlated with DNA extracted from the matched formalin-fixed, paraffin-embedded, surgically resected breast cancer when available. DNA was successfully extracted from 54 of 62 aspirates and all 31 surgical specimens. p53 gene mutations were detected in 10 of the 54 cytology specimens (18.5%) and consisted of base pair substitutions or deletions. Silent or intronic p53 changes were found in five additional aspirates. One of the aspirates had two gene alterations, resulting in a total of six gene changes. Five of these changes were located in introns 6 or 9 and the sixth was a silent (no amino acid change) change in exon 6. p53 Polymorphisms were detected in nine aspirates (16.3%) and were located in codon 47 (one aspirate), codon 72 (six aspirates), and codon 213 (two aspirates). All cases with surgical material available showed identical p53 mutations, alterations, and polymorphisms in the resected tumors compared with those detected in the corresponding aspirates. The results of this study show that DNA suitable for analysis of p53 gene sequence changes can be successfully extracted from ThinPrep-processed breast fine-needle aspirates, and that identical alterations are detected in both the cytology and surgical specimens.

KEY WORDS: Fine needle aspiration; breast cancer; p53 mutation; ThinPrep

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Mutations of the p53 gene are among the most common molecular changes detected in human cancers (1). Experimental studies have shown that functional p53 is required for the *in vitro* cytotoxic action of some chemotherapeutic agents (2). The presence of p53 mutations is associated with an increased chemoresistance to doxorubicin in breast cancer patients (3) and may be involved in the development of multidrug resistance (4). Clinical studies have shown that breast cancers that contain p53 gene mutations are associated with decreased disease-free and overall survival (3, 5-9). These results suggest that the presence of p53 mutations might provide prognostic information and influence the treatment of the breast cancer.

Fine-needle aspiration (FNA) of the breast is a safe, effective method for diagnosing breast cancer with minimal intervention and complications (10, 11). As reviewed by Bédard *et al.*, for the detection of carcinoma, it has a sensitivity ranging from 74 to 97% and a specificity ranging from 82 to 100% (12). ThinPrep-processed and conventionally processed breast FNA have been shown to have similar diagnostic accuracy (12). In addition, immunohistochemistry (13, 14) and molecular analysis (15-17) have been successfully applied to ThinPrep-processed specimens.

Because FNA is often the initial sampling of the tumor, it could be a source of cells for the early detection of p53 mutations. In this study, we examined whether p53 mutations could be detected in the cells present in the residual fluid from ThinPrep-processed breast FNAs. When available, the corresponding paraffin-embedded surgically resected tissue was also analyzed for p53 mutations and the results were correlated.

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MATERIALS AND METHODS

Specimen Acquisition, Clinical History, and Pathology Review

Cytology reports from November 1997 to April 1999 in the files of Mount Sinai Hospital were reviewed. Of the cases diagnosed as positive or suspicious for malignancy, DNA could be extracted from 54 of 62 specimens of ThinPrep processed breast FNA obtained from 62 different women. In cases in which DNA was successfully extracted from the cytology fluid, the surgical pathology records were reviewed to determine whether there was a corresponding breast tumor specimen. Formalin-fixed, paraffin-embedded tissue was available for 31 women. Clinical details and tumor characteristics were obtained from surgical reports. The breast cancers were graded according to the Elston's modified Bloom and Richardson criteria (18). In 30 of the 31 surgical specimens, the tumor was removed after the cytology specimen. On average, the specimen was removed 33 days after the FNA (range, 8 to 72 days). In one case, the FNA was from a tumor recurrence in the scar 6 weeks after the mastectomy.

p53 Molecular Analysis

DNA Extraction: Cytology

After completing the cytological examination the residual preservative fluid (PreservCyt solution, Cytoc Corporation, Boxborough, MA) was stored at 4°C for up to 3 months. The fluid was centrifuged at 4000 g and the supernatant was removed. DNA was extracted from the remaining cells using TriZol (Gibco-BRL, Rockville, MD). DNA extraction was performed according to the manufacturer's instructions for cells grown in suspension. The DNA was stored at 4°C until used for analysis.

DNA Extraction: Surgical Specimens

Sections (5 µm) were cut from the paraffin blocks and stored for up to 2 weeks. Before microdissec-

tion, the sections were dewaxed and stained briefly with hematoxylin. A representative portion of the tumor containing minimal numbers of stromal and inflammatory cells was microdissected and placed in a microfuge tube. The tissue was digested with proteinase K (0.5 mg/mL in 50 mM Tris-HCl, pH 8.5, 10 mM EDTA, 0.5% Tween 20) for at least 48 hours at 55°C (19). The proteinase K was inactivated by heating at 95°C for 15 minutes. The DNA was stored at -20°C for up to 3 wk until further analyzed.

Polymerase Chain Reaction (PCR)—Single Strand Conformational Polymorphism Analysis (SSCP)

A 1-µL aliquot from each sample was added to 14 µL of PCR solution containing 1.5 mM CaCl₂, 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.25 µM concentrations of each primer, 0.1 mM concentrations of each dNTP, 1 U *Taq* DNA polymerase (GibcoBRL, Rockville, MD), and 2 µCi [α -³²P]dATP. The primers and the cycling conditions for each exon are listed in Table 1. The reaction product was run on an 8% nondenaturing polyacrylamide gel and the gel was processed for autoradiography (20, 21). Potential mutations were detected by shifts in band mobility. If there was no band shift, the tissue was considered to have no mutation. For samples showing band shifts, the PCR-SSCP analysis was repeated. In cases in which different band shifts were detected in the cytology and corresponding paraffin-embedded samples, an additional paraffin block was selected, cut, microdissected, and processed as above. Negative controls, paraffin-embedded cells that contained no p53 mutation in the exon examined and a water control to replace the DNA, were included in each analysis. Positive controls for exons 5 to 9 (exon 5, SKBr3; exon 6, T47D; exon 7, colo 320DM; exon 8, MDA-MB468; exon 9, SW480) were also included where appropriate.

p53 Sequencing

The abnormally shifted band was excised from the SSCP gel and the DNA was eluted into water. The DNA was reamplified by PCR using the same

TABLE 1. p53 PCR Primers and Cycling Conditions

Exon	Primer-sense (5'-3') -antisense (5'-3')	Product Size (bp)	Cycling Parameters
4	ATCTACAGTCCCCTTGCCG GCAACTGACCGTGCAAGTCA	296	30 cycles; 50 s at 95°C, 50 s at 55°C, 60 s at 72°C
5	GCTGCCGTGTTCCAGTTGCT CCAGCCCTGTCGTCTCTCCA	294	30 cycles; 50 s at 95°C, 50 s at 58°C, 60 s at 72°C
6	GGCCTCTGATTCTCCTCAGTGA GCCACTGACAACCACCCTTA	199	30 cycles; 50 s at 95°C, 50 s at 55°C, 60 s at 72°C
7	TGCCACAGGTCTCCCCAAGG AGTGTGCAGGTTGGCAAGTG	196	30 cycles; 50 s at 95°C, 50 s at 56°C, 60 s at 72°C
8	CCTTACTGCCTCTTGCTTCT ATAACTGCACCCTTGGTCTC	225	30 cycles; 50 s at 95°C, 50 s at 55°C, 60 s at 72°C
9	GCCTCAGATTCACTTTTATCACC CTTTCCACTTGATAAGAGGTCCC	152	30 cycles; 50 s at 95°C, 50 s at 56°C, 60 s at 72°C

primers and the product was run on a 2% agarose gel. The band was extracted using a QIAquick Gel Extraction Kit (QIAGEN, Chatsworth, CA). The purified DNA was sequenced using a ThermoSequenase radiolabeled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, Ohio) and the sense primer according to the manufacturer's directions, followed by gel electrophoresis and autoradiography. To confirm the mutation, the DNA product was resequenced using the antisense primer. Negative controls were included in each analysis. Cell lines with known mutations in exons 5 to 9 were also included where appropriate. Mutations were compared with those mutations listed for breast cancer in a known p53 database (<http://www.iarc.fr/p53>) (22).

Statistical Analysis

The associations between p53 gene alterations and clinical/tumor variables were examined using the χ^2 or, where appropriate, Fisher's exact test (23). Two-sided *P*-values below 0.05 were considered to be statistically significant.

RESULTS

Histological review of the 31 surgically resected breast tumors showed that they consisted of 29 infiltrating ductal carcinomas not otherwise specified, one invasive ductal carcinoma with lobular features, and one mucinous carcinoma. DNA was successfully extracted from all paraffin-embedded tumors.

Of 62 cytology samples, DNA suitable for p53 sequencing was extracted from 54, yielding an evaluable specimen in 87% of the cases. p53 Gene mutations were detected in 10 of the 54 cytology specimens (18.5%). As shown in Table 2, these consisted of base pair substitutions and deletions. For eight of these 10 aspirates, surgically resected

breast tumor tissue was available for gene analysis. All eight cases showed identical p53 mutations in both the aspirate and the surgically resected tumor. A representative SSCP gel is shown in Figure 1 and the associated sequencing gel is shown in Figure 1B.

Other types of p53 gene changes were found in five other aspirates. One aspirate had two gene alterations resulting in a total of six gene changes. As shown in table 3, five changes were located in introns 6 or 9 and one was a silent change (no amino acid change) in exon 6. For two of these five aspirates, surgically resected breast tumor tissue was available for gene analysis and both of the cases showed identical p53 gene changes in the aspirate and the surgically resected tumor.

p53 Polymorphisms were detected in nine aspirates (16.3%) and as shown in Table 4 were located in codon 47 (one aspirate), codon 72 (six aspirates), and codon 213 (two aspirates). For seven of these nine aspirates, surgically resected breast tumor tissue was available for gene analysis and all seven cases showed identical p53 polymorphisms in both the aspirate and the surgically resected tumor.

The clinical features and tumor characteristics were correlated with the p53 gene status and are summarized in Table 5. DNA suitable for p53 sequencing could be obtained from aspirates of tumors of all three grades. The women whose tumors had a p53 mutation or an intronic change or a silent change were grouped together for these analyses because of the small numbers. There was a significant correlation between a younger age (*P* = .038) or larger tumor size (*P* = .046) with the presence of p53 gene alterations. There was no correlation between the presence of estrogen (*P* = .449) or progesterone (*P* = 0.066) receptors or tumor grade (*P* = .227) and the presence of p53 gene alterations.

DISCUSSION

This study demonstrated that DNA can be extracted from ThinPrep processed breast FNAs. This

TABLE 2. Summary of p53 Mutations

Case Number		Exon	Codon	Sequence Change	Amino-Acid Change
Surgical	Cytology				
20	13	5	*	del 23 bases	
9	7	5	130	C→T	Leu→Phe
10	3	5	175	G→A	Arg→His
36	61	5	183	C→G	Ser→STOP
38	29	6	209	del 2 bases	
13	19	6	220	A→C	Tyr→Ser
17	38	7	232	T→G	Ile→Ser
34	60	7	248	G→A	Arg→Gln
NA	59	8	270	T→C	Phe→Leu
NA	62	9	331	C→T	Gln→STOP

*, deletion (del) starting at nucleotide residue 13041 in intron 4 and involving codons in exon 5.

NA, tissue not available.

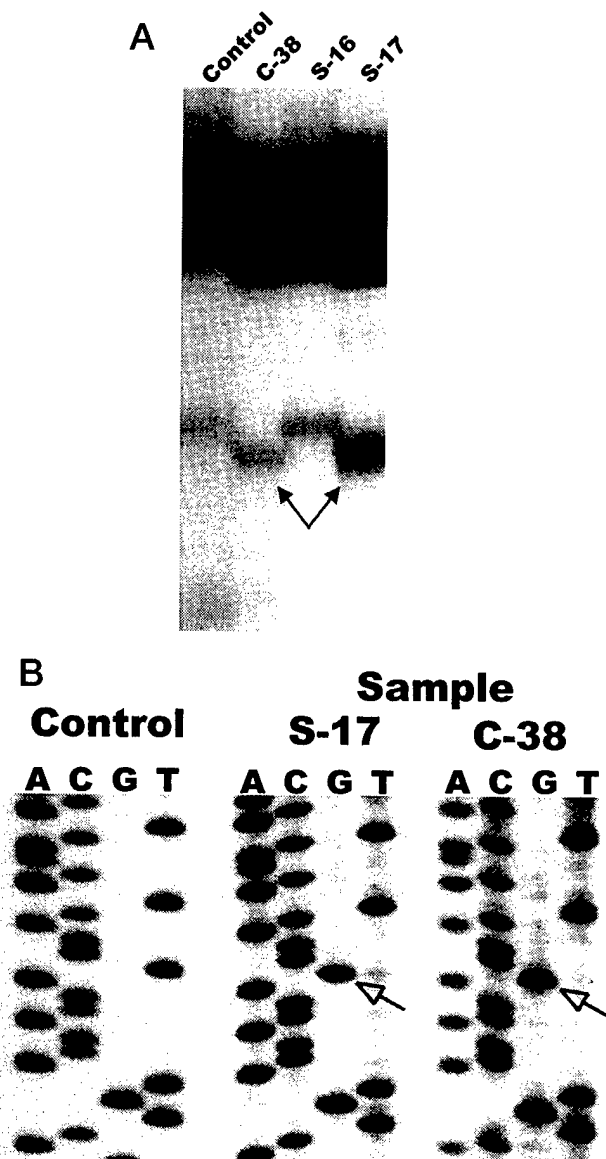


FIGURE 1. A, a representative SSCP gel of p53 exon 7 PCR product from three cases and a negative control (Control). S-16 (surgically resected breast cancer) shows no abnormality. The cytology sample (C-38) and the corresponding paraffin-embedded surgical sample (S-17) show similar band shifts (→). B, the sequencing gel for samples C-38 and S-17 shows a T-to-G base substitution (→). The wild type sequencing pattern (control) in the same region is also shown.

is in keeping with the findings of other groups that have reported successful extraction of RNA or DNA from ThinPrep-processed cytology specimens of breast and cervix (15–17). In addition, the current study showed that the extracted DNA was suitable for p53 gene analysis by PCR-SSCP and sequencing. Using the protocol described above, the mutations detected in exons 4 to 9 were identical to those found in the formalin-fixed, paraffin-embedded, surgically resected breast cancer when this tissue was available for analysis. In contrast, studies assessing p53 immunoreactivity in FNAs and formalin-fixed, paraffin-embedded tumors have shown variable correlations ranging from 73.5 to 93.3% (24–26).

Recent studies have shown that gene alterations detected in paraffin-embedded tissue may be artifacts induced by fixation or processing of surgical specimens (27, 28). Several precautionary steps were undertaken to minimize this possibility. The fidelity of the PCR amplification of DNA extracted from paraffin can be markedly improved by prolonged proteinase K digestion and using small DNA templates (29), so in this study the paraffin-extracted DNA was digested by proteinase K for at least 48 h and the primers were chosen to provide gene sequences of less than 300 base pairs in length. To ensure that the gene alterations were not caused by nucleotide substitutions as a result of *Taq* DNA polymerase misincorporation, all specimens with abnormal SSCP underwent repeat PCR-SSCP to confirm that the change was reproducible. Only those samples that showed similar changes on the repeat PCR-SSCP were considered to have a sequence alteration, which was then confirmed by sequencing. Furthermore, identical alterations were seen in the methanol fixed aspirates and in the corresponding formalin-fixed, paraffin-embedded, surgically resected tumors. This suggests that the p53 alterations identified in this study were genuine.

p53 Mutations were found in 18.5% of patients. This is within the frequency reported for breast carcinoma in other series (8, 9, 30–34). The majority of changes reported for breast cancer have been point mutations (22), and in our series, eight of the 10 mutations (80%) involved base pair substitutions. All mutations, except two (cytology specimens 7 and 13) have been previously reported to occur in breast cancer as listed in a p53 database (22). Silent gene changes were detected in 1.9% of patients, which is similar to the frequency (1.8%) reported by Burns *et al.* (6). In the database examined, there was no report of the silent change observed at codon 224 (cytology specimen 56). No similar comparison could be done for the intronic alterations because the nucleotide position of these types of gene changes is not provided in the database. Codon 47 in exon 4, codon 72 in exon 4 and codon 213 in exon 6 contained known polymorphisms in one, six, and two patients, respectively (1.8, 11.1, and 3.7% of the patients). This is within the range determined for the normal population (35–37). Because the frequencies of mutations and polymorphisms are similar to those shown by others, this suggests that our methodology to detect p53 gene changes is appropriate.

The presence of p53 alterations showed statistically significant associations with larger tumors and younger patient age. No significant association was seen between p53 alterations and tumor grade or the presence or absence of estrogen and progesterone receptors. Other studies examining the associ-

TABLE 3. Summary of p53 Silent and Intronic Changes

Case Number		Location	Site	Sequence Change	Amino-Acid Change
Surgical	Cytology				
NA	56	Exon 6	Codon 224	G→A	Glu→Glu
NA	18	Intron 6	nr 13449	G→C	
NA	55	Intron 6	nr 13964	Del 1 base	
8	35	Intron 6	nr 13964	Del 1 base	
8	35	Intron 9	nr 14755	G→T	
15	5	Intron 9	nr 14766	T→C	

nr, nucleotide residue; NA, tissue not available.

TABLE 4. Summary of p53 Polymorphisms

Case Number		Exon	Codon	Sequence Change	Amino-Acid Change
Surgical	Cytology				
2	36	Exon 4	47	C→T	Pro→Ser
15	5	Exon 4	72	G→C	Arg→Pro
NA	18	Exon 4	72	G→C	Arg→Pro
38	29	Exon 4	72	G→C	Arg→Pro
4	33	Exon 4	72	G→C	Arg→Pro
2	36	Exon 4	72	G→C	Arg→Pro
34	60	Exon 4	72	G→C	Arg→Pro
NA	37	Exon 6	213	A→G	Arg→Arg
31	39	Exon 6	213	A→G	Arg→Arg

NA, tissue not available.

TABLE 5. Patient and Tumor Features

Features	p53 Status		P-value
	Wild-Type	Altered ^a	
Age			
<40	4	2	0.038
40-55	3	7	
56-70	6	1	
>70	7	1	
Tumor Size			
≤2 cm	6	3	0.046
2-5 cm	14	5	
>5 cm	0	3	
Estrogen receptor			
+	13	5	0.449
-	7	6	
Progesterone receptor			
+	13	3	0.066
-	7	8	
Grade			
1	3	0	0.227
2	8	3	
3	9	8	

^a Altered p53 status includes mutations, silent and intronic changes for surgically resected tumors.

ation between these clinical variables and p53 protein accumulation and/or mutations have yielded inconsistent and often conflicting results. For example, Caleffi *et al.* found that p53 mutations occurred in younger patients (38) but other studies have not found an association between age and p53 status (5, 39, 40). The number of patients in the current report is small and may have compromised the statistical power of the study to detect associations.

The use of residual cells from ThinPrep-processed samples has several advantages. First,

the fluid from ThinPrep processing can be stored at 4°C for up to 3 months, before extracting the DNA, as observed in the present study. Second, because only the residual fluid is needed for analysis, the original diagnostic slides do not have to be used. Third, in contrast to paraffin-embedded tissue, which has to undergo proteinase K digestion for at least 48 h before DNA extraction, ThinPrep-processed aspirates can undergo DNA extraction the same day they are obtained. However, there may also be disadvantages to using the residual material from ThinPrep-processing. Not all cases have tumor cells remaining in the residual fluid and thus DNA may not be available for analysis. In addition, if the aspirate contains numerous benign cells admixed with the malignant cells, mutations may be missed (20, 21).

Immunohistochemical staining can be used to detect p53 protein accumulation in either cytological or surgical specimens (24-26) but the immunohistochemical results do not always reflect the presence of underlying genetic changes (33, 34, 41, 42). For example, nonsense mutations will not cause protein accumulation, so these cells will be negative by immunohistochemical staining. In keeping with this, the presence of p53 mutations in the breast cancer was shown to be associated with decreased disease free survival as well as overall survival (5-9, 31), but the presence of p53 protein detected immunohistochemically in the tumor has not consistently been associated with a worse prognosis (7, 8, 42). As molecular analysis of p53 may provide prognostic and treatment information for patients with breast cancer, ThinPrep aspirate is a

suitable alternative to the paraffin-embedded tissue as a source of cells for this type of analysis in patients who will receive neoadjuvant chemotherapy or have unresectable tumors.

In summary, ThinPrep-processed breast FNAs provide DNA suitable for molecular analysis more rapidly than paraffin-embedded tissue. FNAs seem to be a reliable source of cells to determine the p53 gene status, given that identical alterations were detected in both the cytology and surgical specimens examined in this study.

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SIM paper E98/93, Duffy et al, ' Misclassification in a matched...'

Dear David,

I bet you thought you'd never see this paper again. Due to Tom Rohan moving institutes, me moving institutes and the difficulty in getting a variance estimate that works, there has been a two year delay, for which many apologies. I enclose your last letter and the referees' reports.

The paper developed an odds ratio estimate adjusting for misclassification in matched case-control studies with variable matching ratio. After the second round of peer review, the referees were still particularly concerned that our interval estimate was very seat of the pants and our claim that it was conservative was not formally justified. It was recommended that we either find an estimate that could be better justified theoretically or justify our estimate empirically by simulation. We tried the latter and found that our estimate was hopeless. We tried numerous other closed form variance estimates and these all gave similarly unsatisfactory results. Finally, we settled on a simulation-based estimate, using MCMC in BUGS, which not surprisingly gives an answer which is compatible with simulation results.

The paper has been revised accordingly, using the simulation-based interval estimate. I enclose the revised version and hope it is now satisfactory.

All the best, and sorry once more for the unconscionable delay.

Yours ever,

A handwritten signature in black ink, appearing to read 'S. Duffy'.

Stephen W. Duffy, Principal Scientist

MISCLASSIFICATION IN A MATCHED CASE-CONTROL STUDY WITH VARIABLE MATCHING RATIO- APPLICATION TO A STUDY OF c-erbB-2 OVEREXPRESSION AND BREAST CANCER

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**MISCLASSIFICATION IN A MATCHED CASE-CONTROL STUDY WITH
VARIABLE MATCHING RATIO- APPLICATION TO A STUDY OF c-erbB-2
OVEREXPRESSION AND BREAST CANCER**

SUMMARY

We provide a simple analytic correction for risk factor misclassification in a matched case-control study with variable numbers of controls per case. The method is an extension of existing methodology, and involves estimating the corrected proportions of controls and cases in risk factor categories within each matched set. These estimates are then used to calculate the Mantel-Haenszel odds ratio estimate corrected for misclassification. A simulation-based interval estimate is developed. An example is given from a study of risk factors for progression of benign breast disease to breast cancer, in which the risk factor is a biological marker measured with poor sensitivity.

1. INTRODUCTION

There is a considerable literature on the subject of misclassification of risk factors in epidemiological studies. The various methods are reviewed by Bashir and Duffy¹. Methods have been developed for use in the settings of the prospective study², the unmatched case-control study³⁻⁶ and the pair-matched case-control study⁷⁻⁹. In the latter case, Greenland^{7,8} has developed a linear algebraic correction to the estimated numbers of case-control pairs by categories of discrete risk factors, to yield odds ratio estimates which are corrected for the effect of misclassification.

To our knowledge, a readily usable method has not been developed for the corresponding problem of a matched case-control study, a binary risk factor, and a variable number of controls per case. It is the purpose of this paper to develop such a method, prompted by a case-control study of this design in which we encountered a serious deficiency in sensitivity of detection of the risk factor of interest.

We conducted a matched case-control study of breast cancer nested within a cohort of women with benign breast disease, with the aim of establishing risk factors for progression to cancer¹⁰. We have 70 cases and a variable number of controls per case. The risk factor under consideration is the immunohistochemical marker c-erbB-2, as determined by NCL-CB11 antibody testing^{10,11}. Uncorrected risk factor status is shown tabulated by case-control status in Table 1. The sensitivity of the NCL-CB11 antibody was determined in the study by Press et al¹¹, in which a group of 187 archival breast cancers with known c-erbB-2 amplification and expression levels was used to characterize the sensitivity of 28 separate anti-c-erbB-2 antibodies. Tissue sections from the tumours were treated with the antibodies, and overexpression was considered to be present when the breast carcinoma cell membranes showed immunostaining. Sensitivity was calculated as (true positives/(true positives + false negatives)).

2. THE PROBLEM

Suppose we have a matched case-control study with m matched sets. Within each matched set

l (l=1,2,..., m), there is one case and n_l controls. Assume we are interested in the effect of a binary risk factor. Within matched set l, let c_l be the proportion of cases with observed risk factor positive (c_l must equal zero or one), and let r_l be the proportion of controls with observed risk factor positive. If there were no misclassification, we could use conditional logistic regression to obtain the odds ratio estimate of relative risk, or equivalently calculate the Mantel-Haenszel estimate stratified by matched set:

$$OR_{MH} = \frac{\sum (c_l(n_l - n_l r_l)) / (n_l + 1)}{\sum ((1 - c_l)n_l r_l) / (n_l + 1)}$$

Now suppose the determination of the risk factor is subject to error. Clearly, if we perform the statistical analysis using the observed risk factor data, we may obtain seriously biased results¹. If estimates of the error probabilities are available, there is scope in principle for estimating true risk factor prevalences and deriving an odds ratio estimate which is corrected for the misclassification. Greenland⁷⁻⁹ develops a correction method whereby the matrix of observed cell counts is multiplied by the inverse of the product matrix of case and control misclassification probabilities to obtain estimates of the true cell counts. To expand this to the situation of multiple and variable controls per case, it is easier to lay out the calculations in terms of individual cell probabilities rather than in terms of correction by matrix multiplication. Nevertheless, the principle of back-calculation of the true risk factor prevalences within matched sets is essentially the same.

3. CORRECTING THE MANTEL-HAENSZEL ESTIMATE FOR MISCLASSIFICATION

Let RF=0 correspond to risk factor negative status and RF=1 to risk factor positive. Let OF=0 and OF=1 correspond to observed risk factor status. Using Greenland's⁷ notation, we let

$$\pi_{ij} = P(OF = i | RF = j)$$

be the error probabilities for the cases and let τ_{ij} be the corresponding error probabilities for the controls. For the case in any matched set,

$$P(OF = 1|case) = P(RF = 1|case)\pi_{11} + P(RF = 0|case)\pi_{10}$$

Thus the estimated probability that the case is truly positive for the risk factor may be derived by solution of the above equation for $P(RF=1|case)$, as

$$P(RF = 1|case) = \frac{P(OF = 1|case) - \pi_{10}}{\pi_{11} - \pi_{10}}$$

Similarly for a control in any given matched set

$$P(RF = 1|control) = \frac{P(OF = 1|control) - \tau_{10}}{\tau_{11} - \tau_{10}}$$

The probabilities of being truly risk factor negative are easily calculated by subtraction.

From the above, we can calculate the expected numbers of cases and controls positive conditional on the observed numbers by substitution of the observed probabilities, and multiplication of the true probabilities by the numbers of cases (invariably 1) and controls (varying from stratum to stratum) in each stratum. Thus the number of cases positive in stratum l , say, as

$$\frac{c_l - \pi_{10}}{\pi_{11} - \pi_{10}}$$

and the expected number of controls positive as

$$\frac{n_l(r_l - \tau_{10})}{\tau_{11} - \tau_{10}}$$

We can now recalculate the corrected Mantel-Haenszel odds ratio estimate using the expected

true numbers instead of the observed:

$$OR_{MHC} = \frac{\sum_l \frac{(c_l - \pi_{10})(1 - r_l - \tau_{01})n_l}{(\pi_{11} - \pi_{10})(\tau_{00} - \tau_{01})(n_l + 1)}}{\sum_l \frac{(1 - c_l - \pi_{01})(r_l - \tau_{10})n_l}{(\pi_{00} - \pi_{01})(\tau_{11} - \tau_{10})(n_l + 1)}}$$

It should be noted that in the absence of error, this simplifies to the usual Mantel-Haenszel estimate, and in the case of one-to-one matching to Greenland's estimate ⁷.

We can re-express the numerator of the above as

$$\left\{ \sum_l \frac{n_l c_l (1 - r_l)}{n_l + 1} + \tau_{01} \pi_{10} \sum_l \frac{n_l}{n_l + 1} - \pi_{10} \sum_l \frac{n_l (1 - r_l)}{n_l + 1} - \tau_{01} \sum_l \frac{n_l c_l}{n_l + 1} \right\} \\ \times \frac{1}{(\pi_{11} - \pi_{10})(\tau_{00} - \tau_{01})}$$

A similar formula holds for the denominator. Decomposing the summation in this way is useful for computing purposes but has no conceptual value.

4. INTERVAL ESTIMATION

We have gone to some lengths to establish a closed form variance estimate, and tried several forms, but results of simulations indicated that none of our estimates were reliable. We therefore used a simulation technique, Markov Chain Monte Carlo¹², to estimate a 95% credible interval on the logarithm of the corrected corrected odds ratio. This is a Bayesian technique, but we specified uninformative priors to give an estimate which was based only on the likelihood. We used the computer package BUGS¹². The BUGS programme re-expresses the problem as its equivalent conditional likelihood model. The BUGS code is given in the Appendix.

5. EXAMPLE

We return to our matched case-control study of progression of benign breast disease to breast cancer and c-erbB-2 status (see Table 1). The particular antibody test used for this marker in our study has poor sensitivity, given in a large validation study¹¹, external to our study population, as 51%. Specificity is quoted as 100%. From these, and from the total number of samples tested in the validation study, 187, with the quoted proportion of 'gold standard' true positives of 39%, we deduce the validation study data in Table 2. Assuming non-differential error between cases and controls, this corresponds to $\pi_{00}=\tau_{00}=1$, $\pi_{11}=\tau_{11}=0.51$, $\pi_{01}=\tau_{01}=0.49$ and $\pi_{10}=\tau_{10}=0$.

Results uncorrected and corrected for misclassification are shown in Table 3. The correction makes little difference to the point estimate, since although it involves substantial alteration to the estimated prevalence, the alteration applies to both cases and controls. The correction, however, makes a large difference to the interval estimate, since it not only involves correction for the fact that almost half of the true positives are expected to be misclassified, but it also takes into account the fact that the estimated misclassification probability is itself an estimate, with corresponding uncertainty.

6. DISCUSSION

The method proposed here is a simple adaptation of Greenland's approach⁷. It is relatively easy to apply. The estimated corrected cell frequencies can be expressed as a product of a matrix and a vector, both of which are consistent, so that the consistency of the corrected cell frequency estimates follows in the same way as in Greenland⁷. While the formula for the overall estimate is awkward, its component parts are simple, and it is easy to compute. The Bugs programme in the Appendix gives both a point estimate and a 95% confidence interval. A fortran programme for performing the point estimate alone is available from SWD.

When there is 1:1 matching, the point estimate reduces to that of Greenland⁷. In the case of no mismeasurement, the estimate reduces to the usual Mantel-Haenszel estimate. Our method of interval estimation strategy is reliable but dissatisfying in that we have an analytic point estimate and a stochastic interval estimate. Work is in hand to establish a full likelihood solution for both the odds ratio and its variance.

Our example is an interesting one. From Table 3, one can see that there is a large correction to the prevalence estimates (if sensitivity is around 50% and specificity 100%, the true prevalence is likely to be around double the observed). One would normally be reluctant to make any use of a measurement which required such a large correction. It is, arguably, justifiable in this case, that of a biomarker measured by a laboratory test with well-documented false positive and negative error rates.

In principle, this method is extendable to the case of multiple levels of a risk factor and/or the effect of several covariates simultaneously. As before, the most promising approach would be to build on Greenland's method⁷, multiplying a vector of observed proportions in all possible combinations of risk factors by the inverse of a matrix of misclassification probabilities. This is simple in theory, but would give rise to practical problems of dealing with very large matrices if there are numerous potential confounders, and development of variance estimates would be likely to be complex.

In our example, we used both external validation data to calculate the misclassification probabilities. In general, it might be considered preferable to use internal validation, but with two caveats. Firstly, the correction for misclassification is applied multiplicatively, assuming independence of the validation and the main study. Secondly, it is frequently the case that internal resources enable only a small validation or repeatability study to be carried out, whereas results of large and therefore more precise independent validation studies may be available from the literature. Perhaps a reasonable strategy is to use information on the misclassification probabilities from both internal and external sources.

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Table 1: Case-control status by c-erbB-2 status, uncorrected for measurement error

c-erbB-2 status	No. (%) of cases	No. (%) of controls
Negative	62 (89)	235 (86)
Positive	8(11)	39(14)
Total	70	274

**Table 2: True and observed determinations
of c-erbB-2 status from the validation study**

Observed determination	True determination	
	Negative	Positive
Negative	118	34
Positive	0	35

Table 3: Odds ratios and 95 % confidence intervals uncorrected and corrected for measurement error

Correction	Case prevalence	Control prevalence	OR	95% CI
Uncorrected (100% sensitivity, 100% specificity)	11%	14%	0.72	(0.30,1.69)
Corrected (51% sensitivity, 100% specificity)	22%	28%	0.66	(0.18,2.05)

APPENDIX- BUGS programme for point and interval estimation

```

model matchcc;

# read in nos of matched sets with 1,2,3,4,5 contrtols and validation study size

const N1=1,
      N2=6,
      N3=13,
      N4=28,
      N5=22,
      V=187;

var Y1[N1,2], of1[N1,2], p1[N1,2], e1[N1,2], rf1[N1,2], rf11[N1,2],
    Y2[N2,3], of2[N2,3], p2[N2,3], e2[N2,3], rf2[N2,3], rf21[N2,3],
    Y3[N3,4], of3[N3,4], p3[N3,4], e3[N3,4], rf3[N3,4], rf31[N3,4],
    Y4[N4,5], of4[N4,5], p4[N4,5], e4[N4,5], rf4[N4,5], rf41[N4,5],
    Y5[N5,6], of5[N5,6], p5[N5,6], e5[N5,6], rf5[N5,6], rf51[N5,6],
    beta, rf[V], of[V], q, pi[2];

data in "/homef/teresa/ccmisc/cont1.sdat",
      in "/homef/teresa/ccmisc/cont2.sdat",
      in "/homef/teresa/ccmisc/cont3.sdat",
      in "/homef/teresa/ccmisc/cont4.sdat",
      in "/homef/teresa/ccmisc/cont5.sdat",
      in "/homef/teresa/ccmisc/valid.sdt";

inits in "/homef/teresa/ccmisc/match2.in";

{
# external validation study
for (i in 1:V){
    of[i] ~ dbern(pi[rf[i]+1]);
    rf[i] ~ dbern(q);
}
for (j in 1:2){
    pi[j] ~ dunif(0,1);
}
q ~ dunif(0,1);

# matched sets with one control
for (i in 1:N1){
    Y1[i,] ~ dmulti(p1[i,],1);
    for (j in 1:2){
        p1[i,j] <- e1[i,j]/sum(e1[i,]);
        log(e1[i,j]) <- beta*rf1[i,j];

        rf11[i,j] <- rf1[i,j]+1;
        of1[i,j] ~ dbern(pi[rf11[i,j]]);
        rf1[i,j] ~ dbern(q);
    }
}

# matched sets with two controls
for (i in 1:N2){
    Y2[i,] ~ dmulti(p2[i,],1);
    for (j in 1:3){
        p2[i,j] <- e2[i,j]/sum(e2[i,]);
    }
}

```

```

        log(e2[i,j]) <- beta*rf2[i,j];
        rf21[i,j] <- rf2[i,j]+1;
        of2[i,j] ~ dbern(pi[rf21[i,j]]);
        rf2[i,j] ~ dbern(q);
    }
}

# matched sets with three controls
for (i in 1:N3){
    Y3[i,] ~ dmulti(p3[i,],1);
    for (j in 1:4){
        p3[i,j] <- e3[i,j]/sum(e3[i,]);
        log(e3[i,j]) <- beta*rf3[i,j];

        rf31[i,j] <- rf3[i,j]+1;
        of3[i,j] ~ dbern(pi[rf31[i,j]]);
        rf3[i,j] ~ dbern(q);
    }
}

# matched sets with four controls
for (i in 1:N4){
    Y4[i,] ~ dmulti(p4[i,],1);
    for (j in 1:5){
        p4[i,j] <- e4[i,j]/sum(e4[i,]);
        log(e4[i,j]) <- beta*rf4[i,j];

        rf41[i,j] <- rf4[i,j]+1;
        of4[i,j] ~ dbern(pi[rf41[i,j]]);
        rf4[i,j] ~ dbern(q);
    }
}

# matched sets with five controls
for (i in 1:N5){
    Y5[i,] ~ dmulti(p5[i,],1);
    for (j in 1:6){
        p5[i,j] <- e5[i,j]/sum(e5[i,]);
        log(e5[i,j]) <- beta*rf5[i,j];

        rf51[i,j] <- rf5[i,j]+1;
        of5[i,j] ~ dbern(pi[rf51[i,j]]);
        rf5[i,j] ~ dbern(q);
    }
}

beta ~ dnorm(0,1.0E-4);
}

```